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From the Chemistry Department II, Karolinska Institutet, Stockholm.

A Method for the Standardization of Cholecystokinin in Vitro.

By

ERIC H. HULTMAN.

Received 17 November 1954.

In experiments with cholecystokinin-containing preparations of intestinal hormones which cause contractions of the gallbladder, the method of standardization is of prime importance. Several methods for use *in vitro* and *in vivo* have been reported in the literature. Using an *in vivo* method described earlier (HAVERMARK and HULTMAN 1952) in which a cannula is inserted through the papilla of Vater into the common bile duct of the cat under nembutal anesthesia, it was found that some preparations produced intestinal peristalsis concurrently with the contractions of the gallbladder.

In order to analyze this effect, the standardization method in question was complemented with an ordinary intestinal bath technique, which allowed a simultaneous study of the gallbladder and the intestine in the guinea-pig. An ordinary intestinal bath at 37° C was used; it had two cuvettes, each containing about 10 ml of Tyrode's solution, and an arrangement for aeration with oxygen. The contractions in the organs were recorded with two indicators and read off in mm on a scale.

A similar technique was used in 1933 by JUNG and GREENGARD, in tests on both the intestine and the gallbladder of the guinea-pig. In 1953, GERSHBEIN et al. published a standardization method using the gallbladder and the intestine of the guinea-pig in intestinal cuvettes, both organs hanging vertically. In a later investigation by the same authors (1954), the circular layer of

muscle fibres was tested, the intestine then being attached horizontally.

All these authors, as well as earlier workers who have dealt with the problem, have found active cholecystokinin preparations to have a strongly contracting effect on the intestine. In my investigations, the contracting effect on the isolated intestine of the guinea-pig was found to disappear when the cholecystokinin preparations were purified.

Experimental.

After operation of the guinea-pig, the gallbladder and about 1 dm of the distal ileum are transferred to Tyrode's solution at 37° C. A 1.5 cm piece of the intestine is then placed in one of the cuvettes in the intestinal bath, and the gallbladder in the other. The quantity of test solution added is the smallest possible, preferably not in excess of 0.1 ml. The same quantity is used for the gallbladder and for the intestine. Contraction and relaxation of the organs require altogether 10—20 minutes. After a further interval of 5—10 minutes, the solution is changed and a new preparation is tested.

Acetylcholine is used as a test that a definite effect will be produced on the organ in question; in doses of 1—3 γ it causes marked contractions of both the intestine and the gallbladder. After several injections of acetylcholine, experiments with the test solutions can be started. In the present investigation, most of the preparations having an effect on the intestine also proved to cause contractions of the gallbladder; in the same way, the impure cholecystokinin preparations gave rise to intestinal peristalsis.

The effect on the gallbladder varies individually; consequently, the strength must always be correlated with that of a standard preparation. As reported by Ågren (1939), among others, the effect of cholecystokinin on the gallbladder is considerably slower than that of acetylcholine and of histamine. Cholecystokinin results in a slow rise in tonus, which often reaches its maximum only 10—15 minutes after injection into the intestinal bath. When acetylcholine is injected, maximal contractions can be recorded after only a few seconds.

The method was found to give reproducible values, but the gallbladder was noted to exhibit a successive increase in sensitivity to the cholecystokinin preparations during the hours immediately following the operation. This must presumably be ascribed to regression of the paralyzing effect of the operation trauma. A surviving gallbladder can normally be used for 8—10 hours, with new injections every 15—30 minutes. During this interval, the gallbladder (or the intestine) should relax to the

basic value. Thus, it is usually possible to make at least 20 different tests with the same gallbladder. Cramp gradually ensues, resulting in inability of the gallbladder to relax. When this occurs, it can no longer be used.

The intestine has a shorter survival time. The end-result is not a spasm, but a successive decrease in the contractions as soon as after 5—6 hours. This organ is fairly sensitive to the operation trauma, and up to 10 per cent of the specimens fail to react normally. The gallbladder is considerably more resistant. Reddened, hyperaemic, presumably inflamed gallbladders which react poorly are sometimes seen.

In these *in vitro* experiments, an effect on both the intestine and the gallbladder is produced not only by acetylcholine and by impure cholecystokinin preparations, but also by histamine and by acid and alkaline buffer solutions, for example. A specific action on the muscles of the gallbladder is also exerted by ammonium salts, such as the sulphate, the nitrate and the chloride.

In other words, it is fairly easy to induce contractions of the smooth muscles *in vitro*. In order to avoid any of these sources of error, the pH of the test solutions must be carefully adjusted to 7.0; the solutions should also be salt-free as far as possible. The effect of choline and histamine derivatives present in the test solutions is eliminated with atropine and antihistamine (Desentol Leo) respectively, added to Tyrode's solution in a concentration of 1 : 2,500,000.

The intestine-contracting effect which persists — and which is thus due neither to choline or histamine derivatives nor to shifts in the pH — is due to impurities in the cholecystokinin preparations. *When the cholecystokinin preparations are purified, this intestine-contracting effect namely disappears completely* (see table I).

In view of all these sources of error to which the *in vitro* method is subject, the preparations are always controlled on the cat *in vivo*. For this purpose, the cats are anesthetized with nembutal and an incision made in the duodenum; this allows direct observation of the outflow of greenish-black bile from the gallbladder after intravenous injection of the preparation to be tested. In such *in vivo* experiments, non-toxic quantities of acetylcholine, histamine, buffer solutions and ammonium salts have no effect whatsoever, whereas the injection of cholecystokinin results in an adequate flow of greenish bile from the gallbladder.

Table 1.

The Effect of Different Agents on the Isolated Gallbladder and Intestine of the Guinea-Pig in the Intestinal Bath. Weight of Guinea-Pig 525 g (Dark-Haired Male).

Test solution	Readings in mm	Gall-bladder	Intestine
Ordinary technique:			
Acetylcholine 0.003 mg		22	15
Histamine 0.002 mg		25	22
Crude cholecystokinin 1 mg		8	6
Purified cholecystokinin[1] 0.01 mg		7	0
Purified cholecystokinin 0.05 mg		13	0
Ammonium chloride 2 mg		7	0
Acetylcholine 0.003 mg		20	20
After adding Desentol and atropine sulphate (both 1 : 2,500,000) to Tyrode's solution:			
Acetylcholine 0.003 mg		0	0
Histamine 0.002 mg		0	0
Crude cholecystokinin 1 mg		12	8
Purified cholecystokinin 0.01 mg		8	0
Ammonium chloride 2 mg		6	0
Acetate buffer pH 4.70, 1 *M* 2 ml		13	7

[1] Prepared in collaboration with E. JORPES and V. MUTT.

It is possible to note at the same time a relaxation of the sphincter of Oddi when purified cholecystokinin preparations are injected. Preparations causing intestinal peristalsis, on the contrary, often give rise to contraction of the sphincter, thus preventing emptying of the gallbladder.

As far as the choice of experimental animals is concerned, organs from dark-haired male guinea-pigs, weighing between 500 and 600 g, were found to react most satisfactorily. The animals should be starved for at least 24 hours before the experiments. Some spontaneous contractions were occasionally observed in the intestine, but were never seen in the gallbladder.

Summary.

A method is described for the standardization of cholecystokinin *in vitro*, correlated with *in vivo* experiments, and with concurrent testing of the effect on the intestine of impure preparations. The usual intestinal bath technique is used; the gallbladder is placed in one cuvette and a piece of the intestine in another,

both organs being taken from the guinea-pig. The contractions are recorded and read off in mm on a scale.

Histamine and choline derivatives, as well as acid and alkaline buffer solutions, are found to produce contractions of both the intestine and the gallbladder *in vitro*. Ammonium salts also cause contractions of the gallbladder. Even if these sources of error are eliminated, crude preparations of cholecystokinin still cause some intestinal contraction. This effect disappears when the preparations are purified further.

In *in vivo* experiments, the injection of cholecystokinin in the cat under nembutal anesthesia is found to result in a flow of greenish-black bile from the gallbladder, whereas the above-mentioned substances in non-toxic doses cause no reaction.

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From the Department of Surgery, Washington University School of Medicine, St. Louis, Missouri, U. S. A., and the Laboratory of Clinical Physiology, Serafimerlasarettet, Stockholm, Sweden[1].

A Critical Study of Sjöstrand's Method for Determination of Carboxy Hemoglobin, and the Total Hemoglobin Content of the Human Body.

By

HANS DAHLSTRÖM.

Recieved 6 December 1954.

In two papers SJÖSTRAND (1948 a, b) described methods for CO analysis, the determination of CO in alveolar air, the determination of COHb saturation by means thereof, and the determination of the total hemoglobin content of the human body. These methods have subsequently been applied by him and others in investigative work. SIÖSTEEN and SJÖSTRAND (1951) described a method for COHb determinations in blood samples, and some experiments in which their method was applied. CARLSTEN, HOLMGREN, LINROTH, SJÖSTRAND, and STRÖM (1954) did similar experiments from which they concluded that none of the existing methods for CO determination in blood are accurate enough to be used for the exact determination of the relative affinities of CO and O_2 for hemoglobin at such low COHb values as are encountered in Sjöstrand's method for determination of total hemoglobin.

The subject of this paper is the description of a modification of Siösteen and Sjöstrand's method, and its application in rebreathing experiments, similar to the procedure in Sjöstrand's method for determination of total hemoglobin. From the data obtained the apparent relative affinities of CO and O_2 for hemoglobin, under these conditions, could be calculated.

[1] The author's present address.

Methods.

Determination of Low CO Concentrations in Air.

The hopcalite method as developed by SJÖSTRAND and LINDELÖV (SJÖSTRAND 1948 a) was used. The machines were tested by the analyses of prepared dilutions of CO in O_2, which were made by pipetting a carefully measured volume of 96.8 % CO from a storage tank into an evacuated 5,000 ml pyrex flask, see Fig. 1. (A small amount of gas can be measured accurately when confined between mercury columns in a pipette.) The flask was then filled with CO-free O_2 until atmospheric pressure was attained. This mixture was subsequently expelled into a rubber bag by replacement of the gas with water, and then analyzed with the hopcalite method. The hopcalite machines were calibrated with gas of 0.01 % CO. This mixture was made by the Matheson Co. (St. Louis experiments) and by Aga Ltd. (Stockholm experiments). The use of water to displace the gas does not introduce any significant error. The time of exposure of the water to the gas mixture is so short that little gas can enter the water. The calculated error inherent in effecting the dilution of CO with this technique is about 2×10^{-6}.

Determinations of the Hemoglobin Concentration in Blood Samples.

The hemoglobin of an aliquot blood sample was determined spectrophotometrically. In the St. Louis experiments Horecker's method (HORECKER, 1946) was employed, using a Coleman spectrophotometer, calibrated with gasometric analyses. In the Stockholm experiments, a Spekker Photoelectric absorptiometer, Hilger Ilford Filter 605 was used, the blood sample 0.025 ml in 0.04 % ammonium hydroxide solution 2.0 ml. This method was standardized according to KING (1947) and also checked with gasometric analyses.

Determinations of the CO Content of Blood.

The analyses of CO in blood was effected with a modification of the method of SIÖSTEEN and SJÖSTRAND (1951). The principle of the method is the release and extraction of the CO into the gas phase, and the analysis of the gas with the hopcalite machine.

The glass flask described above was used for the extraction procedure, see Fig. 1. The time of extraction, amounts of reagents, etc., were worked out by the repeated performance of the following experiments. About 100 ml of blood was drawn and heparinized and transferred to an evacuating flask and a few ml of physiologic saline was added. It was evacuated by a water suction pump for 15 minutes. A first sample of this blood was then analyzed by the technique to be tested. A second sample of 20 ml was drawn into a 30 ml glass syringe which was made completely gas tight with oil. About 0.20 ml of CO from a gas tank containing 96.8 % CO was carefully measured in a pipette, between columns of mercury. Mercury was brought to the very tip of the pipette, which was connected with the syringe fitting by a small rubber tubing. The gas was transferred to the syringe, leaving some mercury in the

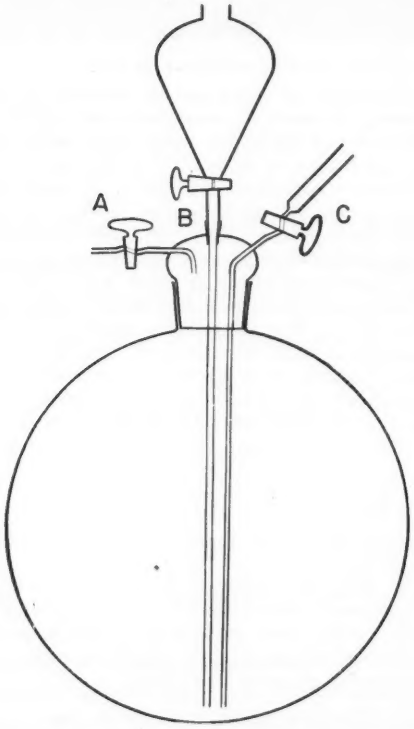

Fig. 1. Glass flask with stopper, used for dilution of CO and extraction of CO from blood samples. — When used for dilution it was evacuated to a few mm Hg., a pipette with the CO attached to A, a rubber bag with CO-free O_2 attached to the other end of the pipette, and stopcock A opened so that the CO was washed into the bottle. Water was added through B and the gas expelled through A into a previously evacuated rubber bag. — When used for blood analyses a plastic covered magnet was first placed in the bottle. The bottle was evacuated, the blood and reagents introduced through the cup C in a way similar to that in the Van Slyke apparatus. The mixture was agitated by a magnetic stirrer, whereupon a rubber bag with CO-free O_2 was attached to A, which was opened; the gas mixture was then expelled and sampled as in the dilution experiments.

syringe fitting. A rubber cap was used to seal the syringe, which was shaken until no gas bubbles were visible in it. This took about 45 minutes. This blood was then analyzed in the same manner as the other sample. The difference in CO content between the first and second samples should be an amount of CO equivalent to the volume added to the second sample.

In other control experiments the CO content was determined on blood equilibrated with 96.8 % CO in a flask for one hour. The expected amount of CO in the blood was calculated from the hemoglobin content and a CO binding capacity of 1.36 ml/gram hemoglobin. The absorption coefficient for CO in blood was assumed to be the same as for CO in water, and the physically dissolved amount calculated accordingly.

The accuracy of the analyses of CO in blood depends upon the actual amount of extracted CO. Therefore, when low COHb concentrations were expected, larger blood samples were used, and the amount of reagents adjusted in proportion.

Rebreathing Equipment and Procedures.

The rebreathing system consisted of mouthpiece, rubber tubings, soda lime canister for CO_2 absorption, and rubber bags which could be exchanged without interruption of the procedure as they were fitted with three-way stopcocks. In order to insure complete mixing in the bags, they were fitted with two openings so that the air would enter and leave at opposite ends. In the St. Louis experiments, respiratory valves directed the air flow. In the Stockholm experiments a powerful blower was used in the circuit instead of valves.

In the St. Louis experiments the O_2 concentration of the gas samples was determined with a Beckman O_2 analyzer, connected in series with the hopcalite machine. In the Stockholm experiments analyses for CO_2 and O_2 were made with a Haldane apparatus.

The CO was introduced into the rebreathing system after measurement of 96.8 % CO in a glass burette which was fitted with three-way stopcocks at both ends. The CO was washed into the inspiratory side of the system with O_2.

The subjects breathed O_2 for a preliminary period. They were then connected to the rebreathing system, which previously had been filled with O_2. In the St. Louis experiments the bag was allowed to shrink during the 15—23 minute rebreathing period. Therefore, the subject had to make a deep expiration when a bag was to be disconnected for analysis of its CO and O_2 content so that a sufficiently large sample could be obtained. The subject was then switched to another bag, which was also filled up with O_2. In the Stockholm experiments a constant flow of O_2 which matched the O_2 consumption of the subject was led into the rebreathing system.

The subject Cr (Table 3) was a normal individual who had abstained from smoking for 24 hours prior to the experiments. Subjects Pn—Gl were examined preoperatively and received the usual preoperative treatment and instruction not to smoke. These individuals were to be operated on for minor diseases. They lay upon a stretcher during the experiments. The subjects for the Stockholm experiments, Sn—Kn, were non-smokers visiting the outpatient service for control of minor disorders. They were seated in a chair during the experiment.

Results.

The calculated CO concentration in 11 preparations of gas mixtures and the CO concentration obtained by analyses of them with the hopcalite machine are included in Table 1. Statistical analysis of the data shows that the values obtained by analysis with the hopcalite machine do not deviate significantly from the calculated values.

Table 1.

Results of Dilution Experiments.

	CO Concentration, Calculated from Dilution. $\times 10^{-6}$	CO Concentration Calculated from Analyses. $\times 10^{-6}$
St. Louis	13	11
	14	13
	47	52
	61	65
	70	66
	80	81
	85	88
	127	123
Stockholm	29	29
	99	99
	104	103
Mean	66.3	66.4

$\chi^2 = 1.656$ $P > 0.99$

The experiments which were performed in order to determine the technical details in the extraction of CO from blood samples, indicated that an extraction time of 1 $^1/_2$ hour was necessary for complete extraction. The standard reagents used were 3 ml. of water and 0.5 ml of 10 % sulfuric acid per ml of blood. The sulfuric acid could be replaced by acid ferricyanide solution with no difference in the results. The first two experiments in Table 2 are the last in this series. The amount of blood used was measured less accurately with a syringe, which may have introduced some error in these experiments.

Table 2.

Results of Determinations of Blood CO Content.

Preparation	Calculated CO Content	Sample Volume	Reagent	Found CO Content	Deviation From Calculated Value
St. Louis	ml	ml		ml	%
Measured amount of CO added to "evacuated" blood.....	0.312*	20	Sulfuric acid	0.303	— 2.9
	0.376*	20	»	0.394	+ 4.8
Blood, equilibrated with 96.8 % CO.	0.438	2	»	0.449	+ 2.5
	0.460	2	»	0.448	— 2.6
Stockholm					
Blood, equilibrated with 96.8 % CO	0.370	2	Ferricyanide	0.367	— 0.8
»	0.370	2	Sulfuric acid	0.373	+ 0.8
»	0.388	2	Ferricyanide	0.389	+ 0.3
»	0.388	2	Sulfuric acid	0.398	+ 2.6
»	0.388	2	»	0.387	— 0.3

Brackets encompass experiments on the same blood samples. *Added amount of CO + amount found in blank.

The results of the experiments with blood saturated with CO are also included in Table 2. It can be seen that the maximal deviation from the calculated values was 2.6 %.

The data for the rebreathing experiments can be found in Table 3. The alveolar pO_2 and pCO were calculated with the assumption that pCO_2 and pH_2O amounted to 40 and 50 mm Hg. respectively. Of course, only the latter figure is needed for the correction of pCO. Hemoglobin was determined spectrophotometrically. "COHb determined" is the result of the analysis of blood samples. "COHb calculated" is calculated according to Haldane's Law I, which states the relations between pO_2, pCO, O_2Hb and COHb, in a system where the hemoglobin is completely saturated with CO and O_2:

$$\frac{[COHb]}{[O_2Hb]} = \frac{MpCO}{pO_2}$$

The factor M was assumed to be 210, and the gas combining capacity 1.36 ml/gram Hb. COHb was obtained by analysis, and the rest of the hemoglobin was assumed to be O_2Hb.

M in Table 3 was calculated according to Haldane's Law I from pO_2, pCO, Hb, and "COHb determined".

Table 3.

Result of Rebreathing Experiments.

Subject	Period of Rebreathing	Alveolar		Hb	COHb Determined	COHb Calculated	M	L
		pO_2	pCO					
	min.	mm Hg	mm Hg	g %	%	%		
Cr	15	610	0.0430	14.7	1.58	1.46	228	227
	19	620	0.1110	15.5	3.91	3.62	228	
Pn	20	538	0.0329	13.9	1.44	1.27	239	227
	15	545	0.0656	13.9	2.73	2.47	237	
Ps	23	627	0.0242	13.9	1.28	0.81	336	353
	21	602	0.0652	13.9	3.62	2.22	347	
Gr	20	556	0.0997	17.3	5.65	3.63	334	403
	18	576	0.1360	16.5	7.65	4.73	351	
Gl	20	481	0.0217	13.9	1.52	0.94	342	263
	20	476	0.0411	13.9	2.56	1.78	304	
Kn	15	549	0.0949	15.2	6.21	3.51	383	218
	15	525	0.1252	14.8	7.45	4.77	338	
Ol	20	610	0.0852	11.8	4.98	2.85	375	273
	18	610	0.1298	11.8	6.75	4.28	340	
Ka	20	622	0.0606	16.9	2.40	2.01	253	229
	20	617	0.1110	16.9	4.17	3.64	242	
Sn	20	568	0.0171	16.1	0.96	0.63	322	219
	20	572	0.0673	16.3	2.81	2.41	246	
An	20	623	0.0177	14.6	1.16	0.59	413	224
	20	611	0.0721	14.4	3.08	2.42	269	

L in Table 3 was calculated according to the formula:

$$L = \frac{\frac{[COHb]_{II}}{100 - [COHb]_{II}} - \frac{[COHb]_{I}}{100 - [COHb]_{I}}}{\frac{pCO_{II}}{pO_{2\,II}} - \frac{pCO_{I}}{pO_{2\,I}}}$$

where I and II denote determinations before and after administration of CO. L is an expression of the relative affinities of CO and O_2 for hemoglobin during the formation of an additional amount of COHb. The error in "COHb determined" does not exceed 2—3 %, with the exception of Sn and An, in whom the blank values were unexpectedly low. Because these two experiments may well be in error, they are treated separately.

The calculated values of M will have errors in common with "COHb calculated", which also will be common with the errors in the method for determination of total hemoglobin. In addition, M will carry the error in "COHb determined" which, as stated above, amounts to 2—3 %.

The error in L can be estimated to remain within $\pm$ 5 %.

A number of single determinations in normal subjects were also obtained. The value of M calculated from these data ranged from 223—333. Further analysis of these data did not yield any additional information, and they are therefore not presented here.

Discussion.

The value of the constant M in Haldane's equation was originally determined by the in vitro equilibration of blood samples with gas mixtures containing CO. SJÖSTRAND (1948 a) refers to a value given by SENDROY, LIU and VAN SLYKE (1929) which was 210 $\pm$ 2.5 %. In their abstract, detailed experimental data were not given. KILLICK (1936, 1948) using her own blood found M = 240. This value was unaffected by acclimatization. On the blood of three men she obtained M values of 233, 246, and 272. ROUGHTON and DARLING (1944) pointed out the difficulty, if not impossibility, of equilibrating blood with CO at low pCO, without obtaining serious, hemic decompositional artifacts. In a review LILIENTHAL (1950) realized this difficulty, but concluded "that the value of M may well be a constant of man".

In Sjöstrand's technique the lungs are assumed to function as tonometer, whereby equilibrium is obtained between alveolar gas and pulmonary venous blood. Several attempts have been made to determine the factor M from in vivo experiments, in which human subjects have been exposed to CO-containing gas mixtures. One difficulty in such experiments is the determination of the time when equilibrium is obtained. Another difficulty is that some of the absorbed CO may not be taken up by the hemoglobin.

KILLICK (1936, 1948) studied the effect of acclimatization upon repeated exposure to CO. The uptake of CO at a given pCO changed during the half-year period. As time passed the amount of COHb formed at a given pCO decreased and the more especially toward the end of acclimatization.

LILIENTHAL, et al. (1945) determined the value of M at low pO_2, using about five hours for equilibration in a chamber. They found values ranging from 182 to 225.

SJÖSTRAND (1948 a) had to rely on indirect evidence for equilibrium as at that time analyses of the CO content of blood at the range he was using could not be performed. He used a small

rebreathing system initially filled with air to which CO was added. Only a short rebreathing period was used so that the pO_2 remained nearly normal. He found that a linear relationship existed between the alveolar pCO and the quantity of CO given to the individual subjects.

In 1951, however, a new method for the determination of CO in blood (SIÖSTEEN and SJÖSTRAND, 1951) was employed, and it was reported that M did not deviate significantly from the anticipated value of 210, and that "for practical purposes at least, it is entirely justifiable to use the calculation suggested by SJÖSTRAND (1944, 1948) for estimating the blood COHb concentration from the CO concentration in the alveolar air". CARLSTEN, et al. (1954), used three different methods for the determination of CO in blood and then calculated the value of M from the data obtained in rebreathing experiments. They found M = 228, 260, and 231, with standard deviations of 45, 59, and 31, respectively, when calculated from the observed *increases* in COHb and pCO after the administration of CO. They point out that "this value of M, signifying the capacity of hemoglobin for binding extra-administered CO, is the relevant one for the calculations entailed in the indirect method for determination of the total amount of hemoglobin, as described by SJÖSTRAND (1948 b)". It is not clear from their paper how the figure M was calculated.

In this paper experiments are described which were aimed at minimizing the analytical errors. The gas analyses were checked with dilution experiments and found to be accurate within a few per cent at CO concentrations about 10^{-4}. The method for determination of CO in blood was also checked, and in 7 check experiments where the blood sample was carefully measured the error did not exceed 2.6 %. In other check experiments, 95 % or more of CO added to a blood sample was recovered in the analytical procedure.

Rebreathing experiments similar to the procedure in Sjöstrand's method for determination of total hemoglobin were performed, during which blood samples were drawn for CO analysis. Such experiments should show whether equilibrium according to Haldane's Law I was actually obtained. This would be the case and M would equal 210, if "COHb calculated" and "COHb determined" were the same. As can be seen from Table 3 this is not so, nor has M any other constant value. We must, therefore, con-

clude that equilibrium, as calculated from Haldane's Law I, was not obtained in these experiments.

If a "gradient" existed from blood to the rebreathing system during the experiment, a high value of M would be expected. This possibility suggested itself particularly with several subjects who were smokers with high blank values and calculated values of M of 300—400. However, as can be seen from the data in Table 3, the COHb values regularly increased on administration of 20—30 ml of CO, and the values of M were somewhat reduced at the same time. This could have been due to a gradient from the rebreathing system to the blood after the administration of CO.

Although the existence of a gradient is suggested by the changes in M, an approximate equilibrium was probably obtained within the rebreathing period, because the changes in M appeared to be rather small when compared with the overall variation in M. This point is of particular interest, because the differences between values of M in one individual are smaller than differences in M between individuals.

The null hypothesis was tested by an analysis of variance according to Fisher. The probability that the variance of the values of M for each individual was due to the same source of variation as the one of the total group of data is less than 0.001. It can, therefore, be concluded that Haldane's Law I cannot be applied to the calculation of COHb under the in vivo conditions used. Incapacity to attain equilibrium between rebreathing system and blood seems to be the most likely explanation for these differences. Variance in the properties of hemoglobin between individuals and within the same individual is also a credible cause.

LILIENTHAL, et al. (1945) obtained a much smaller variation of M from their experiments with low pO_2 and several hours of equilibration. However, analysis of variance of their data again shows that the variation within each experiment (two values of M) is less than the total variation, as in my studies, but no difference between the three individuals could be detected in their material.

The conclusion to be drawn from these findings is that the variation in values of M is less during one experiment than it is between experiments. Determinations of COHb with this technique are obviously unreliable.

Sjöstrand's method for determination of total hemoglobin does not depend on absolute COHb determinations, but on the difference

between a blank value which usually amounts to 0.5—2 %, and a value obtained after administration of CO, which is about 2 % higher.

In Table 3 a value L has been calculated according to a formula mentioned above. This calculation expresses changes in COHb and O_2Hb following changes in pCO and pO_2, as they were observed in two sets of data on each individual. It is conceivable that such changes should be different from what is expressed by M. If, for instance, the very first part of COHb formed (within the range of blank values) is formed with a higher affinity between CO and hemoglobin, then determinations of M at different levels of COHb will be different. A fairly constant value might be obtained at high COHb values, because the first amount of CO is then relatively small and insignificant. But when a certain critical low COHb value is approached wide discrepancies could be anticipated. If the blank values in our studies are high enough to "take care" of this hypothetically particularly active hemoglobin, then it might be possible that the relative affinities of CO and O_2 for hemoglobin when additional CO is taken up is constant, despite wide differences in M.

A comparison of the M values calculated from the blank determination and from the determination after administration of CO shows that the former values are higher than the latter in 6 cases, about equal in 4. This finding is thus suggestive of the possibility that hemoglobin's affinity for CO is higher when the first amount of CO is taken up.

The formula for L was arrived at by the following reasoning. According to Haldane's Law I, $\frac{[COHb]}{100—[COHb]}$ plotted against $\frac{pCO}{pO_2}$ should form a straight line with the slope M (see Fig. 2). From each of our experiments we obtained two points in such a system of coordinates (denoted by I and II in the formula). The two lines from these points to the origin will have slopes which are then two found values of M. The slope of the line connecting the two found points represents L.

Of the 10 values of L (subjects Sn and An included), 6 fall within a range of 219—229. Determinations of total hemoglobin on these 6 individuals would have yielded good results. It is known that determinations on smokers give less accurate results. In our

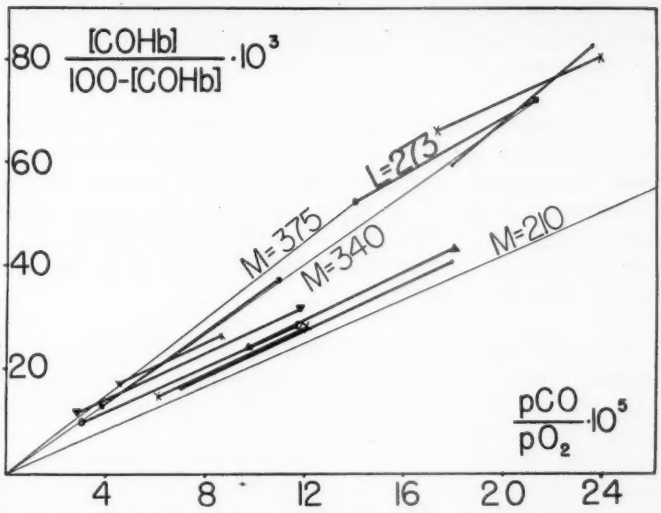

Fig. 2. Representation of the apparent relative affinities of O_2 and CO for hemoglobin on increase of COHb concentration (L, heavy lines). Two values of M, derived from the experiments on Ol, and M = 210, are also represented (thin lines).

material Gr, Kn and Ol have high blank values, probably due to smoking. If they are excluded we are left with 7 values, 5 of which would give good results in calculation of total hemoglobin. In the other individuals there would be considerable inaccuracy in the results because the relative affinities of CO and O_2 for hemoglobin could not be predicted, and due to lack of equilibrium between the rebreathing system and the blood. In our calculations either one of these errors will appear in the value of L. It must, therefore, be concluded that determinations of total hemoglobin with this method occasionally (in this series in 2 cases out of 7) give aberrant results.

Summary.

1. Rebreathing experiments similar to the procedure in Sjö strand's CO method for determination of blood volume were performed.

2. During these experiments blood samples were drawn and analyzed for their CO content with a method which permitted great accuracy even at low COHb values.

3. The results were used for the calculation of a factor L, which is an expression of the relative affinities of CO and O_2 for hemoglobin during the formation of an additional amount of COHb.

4. The results indicate that COHb determinations by analyses of the alveolar air give unreliable results. Determinations of total hemoglobin would occasionally give aberrant results. However, in 5 cases out of 7 the factor L fell within a very narrow range, 219—229. Further investigations will be necessary to explain the reasons for the deviation of the factor L in the other two experiments.

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From the 1st Medical University Clinic, Kommunehospitalet, Aarhus, and the Departments of Physiology and Psychiatry, Aarhus University Medical School, Aarhus, Denmark.

Intracellular Distribution of Brain Phospholipides.[1]

By

V. POSBORG PETERSEN and MOGENS SCHOU.

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Previous work has been concerned with the phospholipide composition of regions of varying histological structure in the central nervous system (BRANTE 1949, JOHNSON, MCNABB and ROSSITER 1948 and 1949). In the present study a different approach has been attempted. Rat brain homogenates were subjected to differential centrifugation, and the concentrations of various phospholipides were determined in each of the cellular fractions. The results obtained from normal rats were compared with those obtained from a series of insulin-treated rats. The reason for including this investigation was the controversial data given in the literature concerning the effect of insulin administration on the content of total lipide phosphorus in the brain (PAGE, PASTERNAK and BURT 1931. RANDALL 1940. MCGHEE, PAPAGEORGE, BLOOM and LEWIS 1951). It was felt that an analysis of different phospholipides in fractionated brain tissue might possibly disclose greater changes than the determination of total phospholipide in whole brain.

Methods.

The animals used were healthy, male albino rats from the State Serum Institute, Copenhagen, a strain inbread through several years and highly homogeneous. They were kept on ordinary laboratory rations and weighed between 170 and 220 g at the time of the experiment;

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A preliminary report of the work was given at the 19th International Physiological Congress, Montreal, 1953.

after the administration of insulin they had no food but could drink water freely. The insulin (equal parts of regular insulin and protamine insulin) was administered as a subcutaneous injection; the usual dose was 1.5 international units, but in some cases additional injections were given if the animal failed to go into coma. The maximal dose administered was 3.5 international units. Most of the insulin rats died spontaneously in coma, but a few were decapitated after having been in coma for about one hour. The average survival time after administration of insulin was four hours and the average time in coma one hour. The onset of coma was characterized by the occurrence of convulsions or by complete lack of corneal reflex. The control rats were decapitated. Each group consisted of 14 animals. Four rats were used in each experiment, two receiving insulin and two serving as controls, and the brains were pooled two by two to give a control and an insulin homogenate. In this way the control and the insulin brains were subjected to exactly the same fractionation procedure during the centrifugation.

Immediately after the decapitation the brains were removed and placed on cracked ice. They were then blotted gently with filter paper, weighed, and homogenized in nine volumes of ice-cold isotonic sucrose (0.25 M in distilled water) in an all-glass homogenizer. Aliquots of the homogenate (H) were taken for nitrogen determination by the micro-Kjeldahl procedure and for determination of phospholipides. The homogenates were fractionated in a refrigerated Servall centrifuge by BRODY and BAIN's (1952) procedure II. Fractions II—R_1 (800 × g for 10 minutes) and II—R_2 (1,500 × g for 10 minutes) were pooled to give a fraction (N), containing nuclei, tissue fragments, whole cells, and mitochondria. Fraction II—R_3 (12,000 × g for 15 minutes) contained mainly mitochondria and is here called M. A last centrifugation (23,000 × g for 30 minutes) yielded a microsome fraction, P, and a final supernatant fluid, S. Each precipitate was suspended in isotonic sucrose and made to a definite volume; total nitrogen and phospholipides were determined on suitable aliquots. The yield of the various fractions, expressed as mg total nitrogen per gm wet tissue, appears from column 6 in Table I.

The lipides were extracted with boiling ethyl alcohol : acetone 1 : 1 by volume, according to SCHOENHEIMER and SPERRY (1934). This has been the usual lipide extraction procedure in our laboratory; but since it had not previously been used for brain phospholipides, it was compared to extraction with chloroform : methanol, 2 : 1 by volume, and the yield was found to be 94 per cent. Total phospholipide, alkali-labile phospholipide and choline-containing phospholipide were determined on the extract as described previously (POSBORG PETERSEN 1950); the relative standard errors of these procedures were two, four, and three per cent, respectively. Sphingomyelin was calculated as the difference between total phospholipide and alkali-labile phospholipide; cephalin was calculated as the difference between total and choline-containing phospholipide, and lecithin as the difference between choline-containing phospholipide and sphingomyelin. All determinations were made in duplicate.

Table I.

Concentration of Total Phospholipide, Phospholipide Fractions, and Total Nitrogen in Brain Homogenate and Cellular Fractions.

Cellular fraction	Total phospholipide	Sphingomyelin	Cephalin	Lecithin	Total nitrogen
			Normal rats		
			mg per g wet tissue		
H[1]	52.7 (47.4—55.4)[2]	5.8 (4.0—7.6)	34.7 (30.0—40.4)	12.0 (7.0—14.3)	18.7 (17.4—22.2)[2]
N	10.0 (8.6—11.4)	1.5 (0.9—2.3)	7.7 (6.3—10.1)	0.8 (—0.9—1.9)	3.0 (2.5—3.5)
M	21.4 (16.2—24.1)	3.4 (1.3—5.4)	14.6 (11.0—17.2)	3.8 (2.7—5.5)	6.0 (4.9—7.6)
P	9.9 (8.4—11.7)	0.9 (0.5—1.4)	6.4 (5.2—7.4)	2.6 (1.5—4.5)	3.7 (2.8—5.0)
S	7.7 (5.5—10.7)	0.0 (—0.4—0.4)	5.8 (3.9—8.6)	1.8 (1.3—2.7)	6.0 (4.8—7.1)
			Insulin-treated rats		
H	49.0 (45.4—52.9)	4.3 (3.1—6.1)	32.2 (28.5—38.4)	12.1 (7.4—15.3)	18.6 (16.7—22.5)
N	10.4 (9.1—12.6)	1.6 (1.3—2.0)	8.1 (7.0—9.4)	0.7 (—0.6—1.7)	2.8 (2.6—3.3)
M	21.2 (16.0—25.8)	4.1 (2.3—7.0)	14.3 (10.8—17.2)	3.3 (1.5—5.0)	6.0 (4.6—7.0)
P	9.5 (7.3—12.3)	0.5 (—0.5—1.3)	5.9 (4.7—7.0)	3.1 (2.1—4.2)	3.5 (2.6—4.3)
S	6.1 (4.5—7.1)	0.0 (—0.5—0.6)	4.6 (3.4—5.5)	1.5 (0.7—3.4)	6.1 (5.0—7.2)

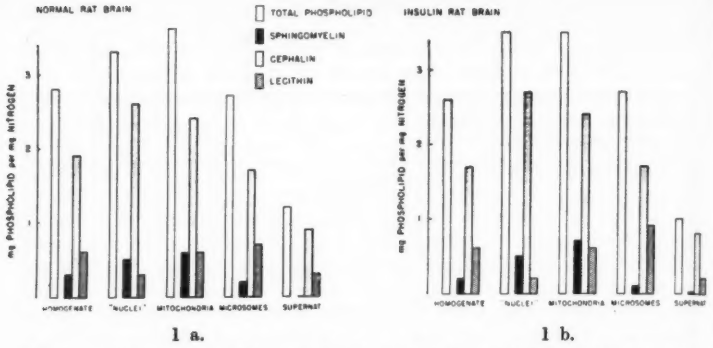
[1] H = homogenate, N = "nuclei", M = mitochondria, P = microsomes, S = supernatant fluid; see text for further details.

[2] The figures in parentheses represent ranges. The brains from 14 animals in each group were pooled two by two: n = 7.

[3] N + M + P + S as percentages of H: normal rats, 100 per cent; insulin-treated rats, 99 per cent.

Results.

Table I shows the distribution of phospholipides in brains from normal and insulin-treated rats. When the total phospholipide contents of the cellular fractions are added, they amount to only about ninety-five per cent of the total phospholipide content of the homogenate; this is due to small, unavoidable losses of material during the separation. Similar discrepancies, but somewhat larger, are found for some of the phospholipide fractions, because they were computed as differences between experimentally determined values.



1 a. 1 b.

Fig. 1 a and b. Concentration of total phospholipide and phospholipide fractions in brain homogenate and cellular fractions. Fig. 1 a: Normal rats. Fig. 1 b: Insulin-treated rats.

The differences between the control and the insulin group are only significant on one point: total phospholipide in whole homogenate ($0.05 > P > 0.02$), but the difference is not significant when the values are expressed on a total nitrogen basis. A comparison of the individual phospholipides in the cellular fractions does not reveal any significant change after insulin treatment, whether the values are expressed on a wet weight or on a total nitrogen basis. Since the total values (total phospholipide in the homogenate) are, theoretically, the sum of these individual values, it must be considered most likely that the lower total phospholipide content per g wet weight of the homogenate from the insulin-treated rats must be due to chance variation. The total nitrogen per g wet tissue was not significantly changed by the insulin treatment in the homogenate and in the cellular fractions, indicating that the water content of the tissue had not changed.

Figure 1 a and b shows the concentration of the phospholipides, expressed per mg nitrogen, in the cellular fractions. It appears that the concentration of total phospholipide is highest in the "nuclei" and mitochondria, lower in the microsomes, and considerably lower in the supernatant fluid than in any of the particle fractions. The sphingomyelin and cephalin concentrations follow in parallel with the total phospholipide, being highest in "nuclei" and mitochondria, lower in microsomes and substantially lower in the supernatant fluid. The distribution of lecithin shows a different pattern; the concentration of this phospholipide is highest in the

Table II.

Statistical Significance of the Differences Between the Phospholipide Concentrations (mg Phospholipide per mg Total Nitrogen) of "Neighboring" Cellular Fractions.

Difference between cellular fractions	Total phospholipide	Sphingomyelin	Cephalin	Lecithin
	Probability			
N—M	> 0.6	> 0.2	> 0.05	*< 0.01*
M—P	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.05*
P—S	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*

Probabilities below 0.05 (printed in Italics) are considered significant. In this table the data from the control and the insulin-treated rats were pooled.

microsomes, with a significant difference between this and the lecithin concentration in the supernatant fluid and in the mitochondria (Table II).

Discussion.

A small, but significant decrease of the total phospholipide content of rabbit brain was found after repeated doses of insulin by RANDALL (1940) and after a single large dose by MCGHEE et al. (1951). Contrary to these findings PAGE et al. (1931) were unable to demonstrate any effect of insulin on the phospholipide content of rabbit brain. In the present study total phospholipide and three different phospholipide fractions were found to remain essentially unchanged in different cellular fractions and in homogenates of rat brain after insulin administration.

The greater part of the phospholipides of the brain are of the non-choline containing type (cephalin). This fraction is a heterogeneous mixture of phospholipides of incompletely known composition, and possible changes in this fraction, not involving the total mass, would not be accounted for with the analytical technique as used here. It is difficult to compare the present quantitative data with those obtained by others, since some results were expressed on a dry weight, others on a wet weight basis; some analyses have been done on whole brain and some on separate samples of grey and white matter; also the phospholipide fractions are defined implicitly by the analytical procedure and therefore vary according to the methods used. If, however, the data from the literature are recalculated on a wet weight basis and for whole brain, a fair

agreement is found between these and the present data: the total phospholipide content is about five per cent of the wet weight; about 65 per cent of the phospholipides are non-choline containing; lecithin constitutes about 20—25 per cent and sphingomyelin 5—10 per cent.

A distribution of the phospholipides as presented here suggests that sphingomyelin and cephalin are mainly localized in the mitochondria and lecithin mainly in the microsomes. It should be emphasized, however, that what is here called "nuclei", mitochondria and microsomes are not pure fractions of these structures. Even in a relatively homogeneous tissue like liver a differential centrifugation cannot yield more than a rather crude separation of the intracellular particles. The brain is a much more complex tissue, containing such different structural elements as the various types of neurones, neuroglia, etc. In spite of these uncertainties BRODY and BAIN (1952) have presented histological and biochemical evidence showing that the mitochondria- and the microsome-fractions obtained from brain by their procedure II contain mainly particles that are structurally and metabolically equivalent to the corresponding particles isolated from liver. The supernatant fluid contains only a few small particles, while the nuclear fraction is contaminated with mitochondria; it is therefore not surprising that this fraction does not differ significantly from the mitochondria fraction.

Summary.

Total phospholipide, sphingomyelin, cephalin, and lecithin have been determined in rat brain homogenate and in the various cellular fractions obtained by differential centrifugation in isotonic sucrose.

It was found that the concentration of all phospholipide fractions, expressed per mg total nitrogen, was considerably lower in the supernatant fluid than in any of the particle fractions. Sphingomyelin and cephalin were localized mainly in the mitochondria (large granules), whereas the concentration of lecithin was highest in the microsomes (small granules). The phospholipide composition of the nuclei could not be established satisfactorily, since this fraction was too impure.

Comparison between brains from normal rats and rats in insulin coma revealed no differences in the phospholipide composition of the various cellular fractions.

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From the Department of Physiology, Kungl. Veterinärhögskolan, Stockholm 51.

The Response Spectrum of Taste Fibres in the Cat: A Single Fibre Analysis.

By

MELVIN J. COHEN[1], SUSUMU HAGIWARA[2] and YNGVE ZOTTERMAN.

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In a recent paper, LILJESTRAND and ZOTTERMAN (1955) observed that certain taste fibres in the cat chorda tympani nerve were stimulated by a flow of water over the tongue. The water response was, in many respects, similar to that observed in taste fibres of the frog glossopharyngeal nerve (ZOTTERMAN 1949, ANDERSSON and ZOTTERMAN 1950).

In the previous investigation the fibres responding to water were differentiated from those responding to hypertonic salt solutions though both kinds responded to acids. It is known from the observations of PFAFFMANN (1941) that many mammalian taste fibres respond to more than one chemical stimulus and it was felt that the response spectrum of the mammalian "water" fibres should be more rigidly defined. The mechanism by which a flow of water across a receptor membrane elicits nerve impulses in an associated afferent neurone is also of interest, particularly when it is known that the mechanical factor is not responsible. Knowledge of the necessary ionic exchanges involved in "water" receptor stimulation may provide additional information concerning the chemical requisites of excitation and inhibition.

[1] Postdoctoral fellow of the U.S. National Science Foundation. Address after September 1, 1955: Dept. of Biology, Harvard University, Cambridge 38, Mass. U. S. A.

[2] On leave from Physiological Department, Tokyo Medical and Dental University.

It was also desired to evaluate the possible physiological rôle of the "water" fibre and to determine how the water response may be integrated into peripheral and central aspects of taste sensation. This requires that the adequate stimuli of other taste fibres, in addition to the "water" fibre, must be well defined.

The present investigation is therefore concerned with a single fibre analysis of the cat chorda tympani taste fibres in the hope that certain of these questions may be clarified.

Materials and Methods.

A total of 28 cats were used in this study. The animals were anaesthetized by intramuscular injection of a chloralose-urethane solution (0.05 g chloralose and 0.25 g urethane in 7 ml of Ringer's solution per kg body weight).

In order to expose the central portion of the right chorda tympani nerve after its exit from the bulla, the right mandible was resected allowing the chorda tympani to be dissected free and cut approximately 1 cm central to its junction with the lingual nerve.

The lingual nerve was cut just central to its junction with the chorda tympani and dissected free for approximately 7 mm distal to this junction. The cut central end of the lingual nerve together with the attached central portion of the chorda tympani were lifted into a small lucite chamber which was positioned adjacent to the exposed nerves. After filling the chamber with Ringer's solution at 37° C, the epineural sheath of the chorda tympani nerve was removed under a dissecting microscope and the nerve divided into 5 or 6 bundles. Each bundle was then dissected until a few or single fibre preparation was obtained.

For recording the action potentials from chorda tympani nerve fibres, most of the Ringer's solution was drawn out of the chamber and paraffin oil at 37° C was layered over the remaining Ringer's. The nerve bundle was placed across a platinum wire electrode which was then lifted up into the paraffin. An indifferent electrode made contact with the remaining Ringer's solution in the chamber. The electrodes were connected to a 4-stage, RC coupled preamplifier and the amplified action potentials then recorded on a cathode ray oscilloscope. A loudspeaker was also used to monitor the nerve activity.

Solutions were initially applied to the tongue with a syringe in order to locate the 5×6 mm region innervated by the particular nerve fibre under observation. A special burette was then fixed rigidly in position with its tip approximately 5 mm above the predetermined sensitive region of the tongue. Fifteen ml quantities of the test solution were used for each trial and the burette and tongue were flushed with Ringer's solution between each application. It required 3 seconds to empty 15 ml of solution, providing an average flow rate of 5 ml per second. The flow

was not constant during any single trial using this method because the height of the fluid column dropped to zero as the burette emptied. However, the rate of flow followed the same time course in all the trials thus enabling a comparison of the effects of different fluids. By making the hole in the stopcock barrel and the burette nozzel the same diameter, no air was trapped between the barrel and the nozzel thus preventing irregularities of fluid flow from one trial to the next. When a constant velocity flow was required, as in the adaptation experiments, a 2 liter reservoir of the test fluid was connected to the burette. The rate of flow from the reservoir was adjusted to equal that from the burette at the 15 ml level. Thus the height of the fluid column in the burette could be maintained relatively constant for 30 seconds or longer, thereby providing a flow of fluid over the tongue at constant velocity. The relatively slight drop in the height of the fluid column in the reservoir can be neglected under these conditions.

As the surface of the exposed tongue is at a temperature of 26—28° C, all solutions were maintained at 26—28° C by immersion in a water bath in order to avoid stimulating thermoreceptors whose axons run in the chorda tympani nerve (DODT and ZOTTERMAN 1952).

The hydrogen ion concentration of all solutions was determined at the end of each experiment using a Beckman model G pH meter. Distilled water ranged from pH 5.7 to 7.1. The various inorganic and organic salt solutions ranged from pH 6.0 to 7.7, while the quinine solutions extended from pH 6.5 to 7.7. The solutions used specifically to examine the effect of high hydrogen ion concentration were unbuffered in order to avoid any possible effect of the buffering ions. The pH of these solutions was determined immediately after they were used.

By placing the tongue outside of the buccal cavity and rinsing it frequently with Ringer's solution, it was kept relatively free of saliva. Any possible buffering action by the saliva was avoided by this procedure.

Results.

Whole nerve response. A continuous discharge of impulses is observed when recording from the entire chorda tympani nerve. Fig. 1 shows the effect of various solutions flowed over the tongue upon the impulse traffic in the chorda tympani. Distilled water and 0.5 M NaCl each cause a prolonged increase in activity. Ringer's solution or 0.28 M sucrose in Ringer's solution elicit no change in the level of spontaneous activity. A solution of 0.009 M quinine hydrochloride made up in distilled water causes a prolonged massive discharge of greater magnitude than the response caused by distilled water alone (cf. Fig. 1 A & E). Hydrochloric acid in Ringer's solution at pH 2.5 or below causes a response similar to that evoked by quinine hydrochloride.

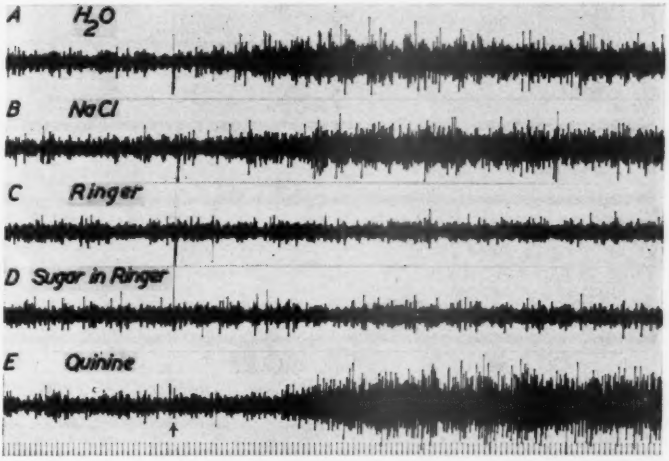

Fig. 1. Response of the whole chorda tympani nerve to various solutions flowed over the tongue. A, distilled water; B, 0.5 M NaCl; C, Ringer's solution; D, 0.28 M sucrose in Ringer's solution; E, 0.009 M quinine hydrochloride in water. The flow of solution continued beyond the end of each record. Note spontaneous activity preceding stimulus artifact and the absence of response in C and D. Time marker 50 cycles.

The magnitude and duration of the water response indicate that it should be considered on a level of physiological validity similar to the responses evoked by more well known chemoceptive stimuli.

Single fibre analyses.

"*Water*" *fibre.* In 12 cats we obtained 14 preparations containing a functional single fibre which responded to distilled water. Fig. 2 illustrates the water response in 2 different single fibres. An absence of spontaneous activity is characteristic of this fibre type although they may occasionally be active at frequencies of 1 to 3 per second in the absence of stimulation. The response to water appears dependent upon an actual flow of water over the tongue. The water response persists for a short time after the flow has ceased but the decline in frequency is more rapid than in other chemoceptive fibre types following cessation of flow of their adequate stimulating solutions. The dependence upon rate of flow over the tongue can not be due to any mechanical effect because a similar flow

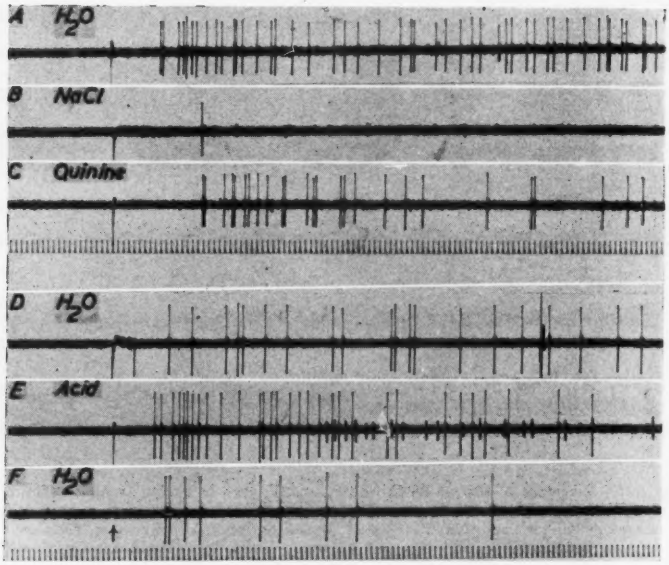

Fig. 2. Response of single chorda tympani "water" fibres to various solutions flowed over the tongue. Records A—C from same single fibre: A, distilled water; B, 0.5 M NaCl; C, 0.02 M quinine hydrochloride in Ringer's. Records D—F, from a different single fibre, illustrate the depressing effect of acid upon the subsequent water response: D, distilled water; E, HCl in Ringer's solution at pH 1.5; F, distilled water applied 30 sec. after the acid. Arrow in F indicates when first drop hit tongue. This is shown by downward artifact in other records. The flow of solution continued beyond the end of each record. Note absence of spontaneous activity. Time marker 50 cycles.

of a number of other solutions, including Ringer's, does not evoke such a response.

In addition to their response to water, these fibres are also characterized by a sensitivity to quinine hydrochloride (Fig. 2 C), and to mineral acids such as HCl (Fig. 2 E). The quinine threshold varies from fibre to fibre and ranges from 0.009 M to 0.002 M. The threshold for HCl ranges from pH 2.0 to 2.5.

The application of HCl at pH 2.5 or lower has a definite depressing effect upon the subsequent response to water (cf. Fig. 2 D & F). Both the initial frequency and the duration of the water response are decreased by previous application of acid. The acid depression lasts for approximately 10 minutes although some preparations did not show a complete recovery of water sensitivity for

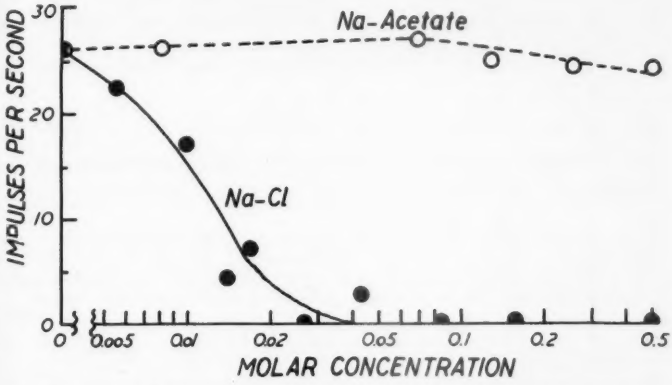

Fig. 3. Response of a single chorda tympani "water" fibre to varying concentrations of NaCl (solid curve) and the response of a different single "water" fibre to sodium acetate (broken curve). Each point represents the number of impulses during first second after initiation of the response. As the magnitude of the water response differed slightly between the 2 fibres, the sodium acetate curve was adjusted by taking the water response of the fibre tested with NaCl as 100 per cent. Note that the activity is completely depressed by NaCl concentrations above 0.03 M, while sodium acetate elicits a response approximately similar to water.

as long as 30 minutes after application of HCl at pH 1.5. It is interesting to note that the application of acid does not affect subsequent responses of the same fibre to acid or quinine solutions. Acid depression extends only to the water response indicating that either separate receptors or distinct loci on the same receptor respond to water and acid respectively.

These fibres do not respond to NaCl solutions above a concentration of 0.03 M nor to a 0.28 M sucrose solution made up in Ringer's solution. However, a 0.28 M sucrose solution in water elicits a response similar to that evoked by water alone, indicating that low osmotic pressure *per se* is not important in eliciting the water response, thus confirming LILJESTRAND and ZOTTERMAN (1955).

Figure 3 illustrates the response of one "water" fibre to varying concentrations of NaCl and of another "water" fibre to varying concentrations of sodium acetate. It appears that NaCl in concentrations above 0.03—0.04 M depresses the activity of the "water" fibre. Other salts in equimolar anion concentration such as NaBr, NaI, $NaNO_3$, $CaCl_2$, KCl and choline chloride have the same effect as NaCl. Sodium acetate, in contrast, has very little depres-

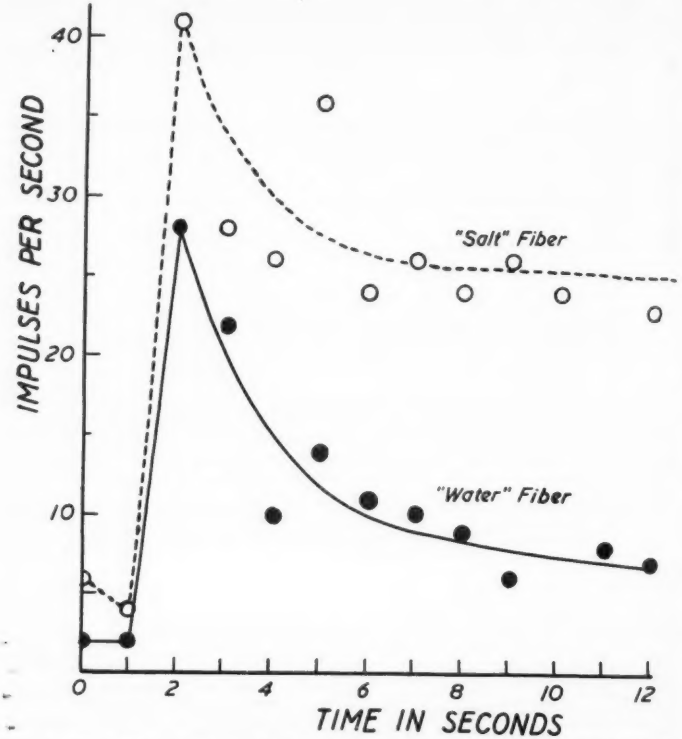

Fig. 4. The solid curve shows the adaptation of a single chorda tympani "water" fibre to water flowing over the tongue at a constant rate of 5 ml per sec. The broken curve shows the adaptation of a single chorda tympani "salt" fibre to 0.5 M NaCl flowing over the tongue at a similar rate. Each point represents the number of impulses during a one second interval.

sing action upon the "water" fibre. Concentrations approaching 0.5 M still elicit responses similar to that evoked by distilled water. Sodium formate acts similar to the acetate solutions.

Occasionally a single "water" fibre responded slightly to NaCl solutions at concentrations of 0.5 M or above. The response to strong salt solutions had a relatively long latency and consisted of a brief grouped discharge at a frequency less than one third of the maximum water response. A similar occasional hypertonic salt response was observed in frog "water" fibres by ANDERSSON and ZOTTERMAN (1950).

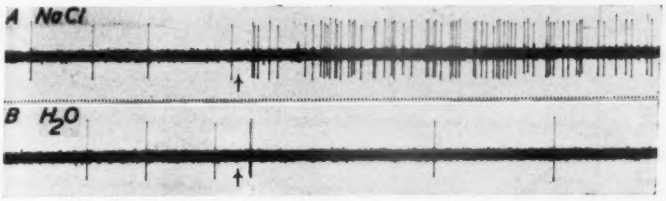

Fig. 5. Response of single chorda tympani "salt" fibre to various solutions flowed over the tongue. A, 0.5 M NaCl; B, distilled water. Arrows mark approximately when first drop hit tongue. The flow of solution continued beyond end of each record. Note presence of spontaneous activity. Time marker 50 cycles.

The adaptation of a single "water" fibre to a constant flow of distilled water over the tongue is shown in the graph of Fig. 4. Adaptation occurs relatively rapidly but the frequency never decreases to the pre-stimulus level.

"Salt" fibre. This fibre type is characterized by its sensitivity to hypertonic salt solutions. It also responds to acid in a manner similar to the previously described "water" fibre but does not respond to distilled water (Fig. 5). Application of acid does not depress subsequent responses to salt in the same fibre. In contrast to the "water" fibres, this fibre type was usually spontaneously active at an average frequency of 5 impulses per second.

Figure 6 shows the response of a single "salt" fibre to varying concentrations of NaCl and choline chloride. The threshold to NaCl is about 0.1 M. Other inorganic chloride salts such as KCl and $CaCl_2$, in equimolar chloride concentration, all evoke responses similar to NaCl. A series of different sodium salts was examined to determine the effect of the anion on "salt" fibre activity and it was found that Cl^- was most effective; the Br^-, I^- and acetate ions elicit slightly lower responses than the Cl^- ion, while NO_3^-, below a concentration of 0.5 M, does not stimulate at all.

Choline chloride had a remarkably strong stimulating effect on the "salt" fibre as depicted in the broken curve of Fig. 6. It is seen that concentrations as low as 0.009 M elicit a response similar to that evoked by 0.5 M NaCl. This is in contrast to the "water" fibres which respond to choline chloride just as to any other chloride salt. The high sensitivity to choline chloride was observed in most "salt" fibres although occasionally one was obtained which

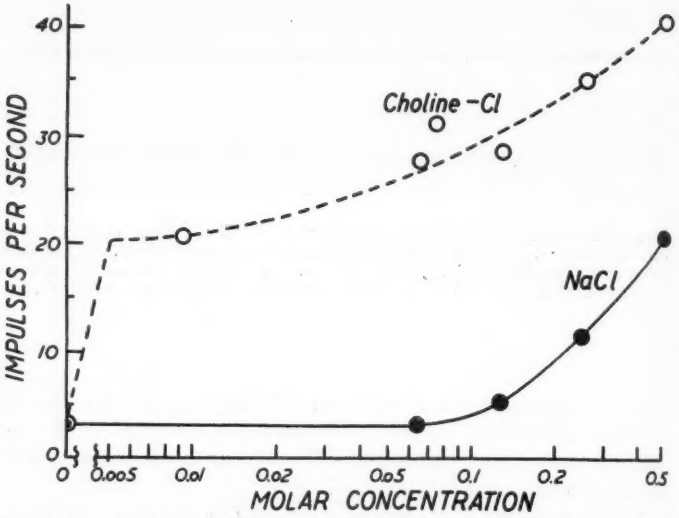

Fig. 6. Response of single chorda tympani "salt" fibre to varying concentrations of NaCl (solid curve) and choline chloride (broken curve). Each point represents the number of impulses during first second after initiation of the response. Note the extreme sensitivity to choline chloride.

did not show such a hypersensitivity but responded to choline chloride in a manner similar to any other chloride salt.

The adaptation of a single "salt" fibre to a constant flow of 0.5 M NaCl over the tongue is shown in Fig. 4. It is seen that this fibre type has a much slower rate of adaptation than the "water" fibre. The "salt" fibre response does not appear as dependent upon the rate of flow of solution over the tongue as the "water" fibre. This is demonstrated by the longer persistence of the "salt" fibre response after flow of the effective stimulus has stopped. The "salt" fibre response is abolished by a Ringer's solution rinse. This fibre type appears similar to the salt-acid fibre described in the cat by PFAFFMANN (1941).

"Quinine" fibres: Single functional fibres were observed which responded primarily to quinine hydrochloride solutions. The threshold was approximately 0.002 M. As seen in Fig. 7, these fibres are relatively insensitive to acid, as they respond very little to HCl at pH 2. There was no response to 0.5 M NaCl, distilled water or 0.28 M sucrose made up in either Ringer's solution or water.

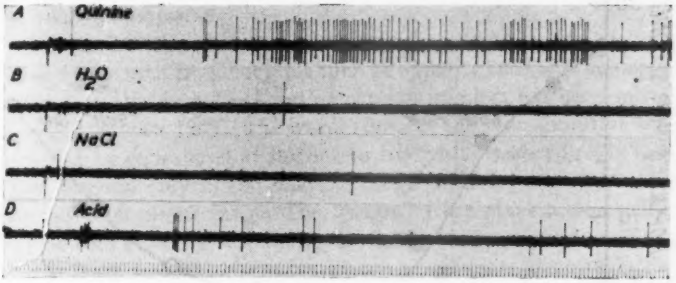

Fig. 7. Response of a single chorda tympani "quinine" fibre to various solutions flowed over the tongue. A, 0.009 M quinine hydrochloride in Ringer's; B, distilled water; C, 0.5 M NaCl; D, HCl in Ringer's solution at pH 2. Note the relatively small response to acid shown in D. Time marker 50 cycles.

Choline chloride also was ineffective. These "quinine" fibres differ from the quinine-acid fibres described by PFAFFMANN (1941) in that they are relatively insensitive to strong mineral acids.

"Acid" fibres: On 2 occasions, functional single fibres were obtained which responded only to mineral acids at pH 2.5 or lower. They did not respond to any other test solutions previously mentioned. These fibres are probably similar to the acid fibres described by PFAFFMANN (1941). This author described a "transient response" in acid fibres elicited by applying distilled water to the tongue with a brush. In contrast we did not observe any "acid" fibre response to distilled water flowed over the tongue.

Discussion.

Physiological rôle of the "water" fibre. The magnitude and duration of the water response in certain fibres of the chorda tympani nerve indicate that this response should be considered as a physiologically valid occurrence. The existence of "water" fibres provides a means of extending the range of peripheral saline sensitivity beyond the concentration limits covered by the "salt" fibres. This is indicated in the composite graph of Fig. 8. As the salt concentration falls below 0.1 M the "salt" fibres are no longer sensitive to changes in saline concentration. If the water fibres were not present, then no means would exist for the peripheral discrimination between distilled water and salt solutions

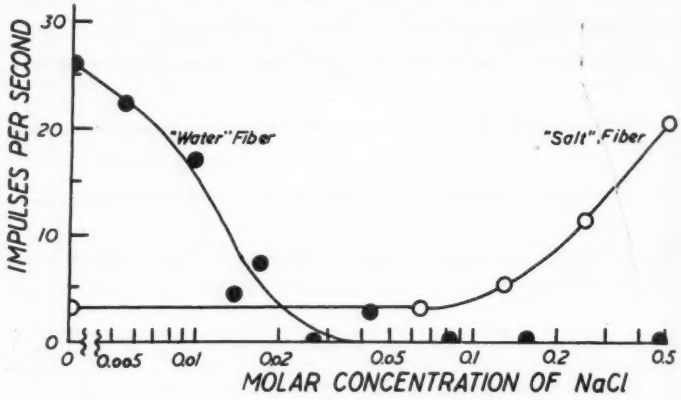

Fig. 8. Response of a single chorda tympani "water" fibre (solid circles) and a single "salt" fibre (open circles) to varying concentrations of NaCl. Each point represents number of impulses during the first second after initiation of the response.

below approximately 0.1 M. The presence of the "water" fibre, however, may provide a means for discrimination in the 0.1 M salt to distilled water range by the following means. The region between 0.1 M salt (where the "salt" fibre response ends) and 0.03 M salt (where the "water" fibre response begins) could be signalled by the spontaneous activity of the "salt" fibre and the lack of activity in the "water" fibre. Saline concentrations below 0.03 M are indicated by the progressively increasing activity of the water fibre. It may be that discrimination is lacking within the region extending from 0.03 M salt to distilled water and that this segment of the saline range is interpreted centrally simply as "water". The presence of "water" fibres in addition to "salt" fibres therefore appears to extend saline sensitivity from the extreme hypertonic region to the extreme hypotonicity of distilled water, thereby covering the entire physiologically significant saline range.

SKRAMLIK (1926) reported that in humans, salt solutions below 0.03 M taste sweet and that "saltiness" appears only with concentrations above 0.03 M. Although it may be dangerous to generalize from cat receptor-threshold to human sensation, it is nevertheless intriguing that a salty taste is evoked in humans only when a saline concentration is reached where the cat "water" fibres are completely depressed. This may indicate that the salty sensation evoked by

relatively low salt concentrations is due to the absence of activity in "water" fibres.

Discrimination within an entire stimulus range by two distinct fibre types with frequency maxima at opposite ends of the range is similar to the way the thermo-reception problem has been solved (see ZOTTERMAN 1953). The warm fibres of the thermoceptive system can be considered analogous to the "salt" taste fibres in that both respond only in the relatively high intensity region of their respective stimulus range. The "water" fibre plays a rôle similar to the cold fibre by responding in the low intensity region. Thus for at least two exteroceptive systems, adequate discrimination within the entire physiologically significant stimulus range is beyond the capability of a single receptor; the general solution of employing two distinct fibre types which respond in opposite regions of the sensitive range has been evolved as a solution for this problem.

Fibre type and taste sensation. The observation that acid as well as quinine stimulates the "water" fibre raises the question of specificity of fibre types. PFAFFMANN (1941) found that acid stimulated, with equal facility, all taste fibre types which he observed in the cat. He took this as evidence that the acid taste results from a general stimulation of all taste fibre endings and emphasized the point that taste sensations result from the total input pattern in several fibre types rather than the activity of a single specific kind of fibre. In general our results bear out the conclusions of the previous author with respect to the importance of input pattern, however, certain modifications seem indicated.

We have observed 4 distinct taste fibre types in the cat chorda tympani nerve in contrast to the 3 types described by PFAFFMANN in the cat glossopharyngeal and chorda tympani nerves. The "water" fibre described in the present study may very likely be the same fibre designated as the quinine-acid fibre by PFAFFMANN. Certainly the quinine and acid thresholds are quite similar. The previous author, however, did not state whether or not he tested this fibre type with distilled water or low concentration saline solutions. Also the method of stimulation used by this investigator consisted in applying solutions with a small brush and this may have prevented observation of the water response because of its dependence upon the rate of flow of solution over the tongue. The frequent application of acids below pH 2.5 may also have hindered observation of the water response due to the prolonged depressing

action acid exerts on the "water" receptor endings. The transient occasional response to distilled water occurring in the specific acid fibres described by PFAFFMANN (1941) can not be considered the same as the water response discussed in the present paper for the following reasons: 1) It was of relatively low frequency and brief duration, 2) it occurred in a fibre type not responsive to quinine or to sodium acetate, both of which stimulate the "water" fibre described in the present paper, 3) PFAFFMANN states that the acid fibre was sensitized to water by previous acid stimulation while the presently described "water" response is markedly depressed by acid.

Despite the fact that quinine and acid can stimulate the fibre type mediating the water response, it is felt that in life this fibre type is most frequently activated by a flow of aqueous solution over the tongue and thus the designation of "water" fibre best describes its physiological rôle.

A fibre type responding primarily to quinine has not been described previously. The specific "quinine" fibre observed in this study differs from PFAFFMANN's quinine-acid fibre by its very low sensitivity to acid. The response to acid shown in Fig. 7 D, while small, is greater than usually observed in this fibre type. Often no response was seen to HCl at pH 1.5. It is important to note that the "quinine" fibre does not respond to distilled water. We did not begin applying quinine to the tongue in preparations not responding to any of the other stimuli until the last two experiments of this study. However, when quinine was applied to such preparations (which we previously considered to have no functional fibres) a specific quinine response was often demonstrated. This indicates that there may be a relatively large number of the specific "quinine" fibres in the cat chorda tympani nerve. It is interesting to note that PFAFFMANN (1953) described a greater quinine sensitivity in cat taste receptors as compared to those of the rat and rabbit.

It is apparent that even the so-called primary taste sensations are built up from the composite input pattern of several different taste fibre types. Table I summarizes the possible relationship between impulse pattern and taste sensation for the fibre types described in this study.

In this study no response to sweet solutions was seen in few or single fibre preparations. In animals such as the dog where specific "sweet" fibres have been demonstrated (ANDERSSON,

Table I.

Fibre type response.

Stimulus	"Water" fibre	"Salt" fibre	"Acid" fibre	"Quinine" fibre	Sensation evoked
H_2O (salt <0.03 M)	+	0	0	0	→ Water
NaCl (0.05 M)	0	+	0	0	→ Salt
HCl (pH 2.5)	+	+	+	0	→ Sour
Quinine	+	0	0	+	→ Bitter

LANDGREN, OLSSON and ZOTTERMAN 1950) they would readily fit into the above scheme. If it is assumed that sweet substances are usually carried in an aqueous solution, then a sweet sensation would be evoked when the "sweet" and "water" fibres were simultaneously stimulated.

Even with stimuli which activate only one fibre type respectively such as water and NaCl, the impulse pattern still must be considered important in that the *lack* of response in the other taste fibres is necessary for the arousal of a water or salt sensation. It appears that the activity of the 2 fibre types which respond only to one stimulus, *i. e.*, the "quinine" fibre and "acid" fibre, dominate the "water" fibre activity centrally during simultaneous stimulation. Thus a bitter taste results when the "quinine" and "water" fibres respond simultaneously; a water taste is aroused when the "water" fibres alone are stimulated.

Receptors involved. The observation that different stimuli initiate impulses in the same single fibre raises the problem of receptor specificity. PFAFFMANN (1941) mentioned the possibility that different receptors may be connected to the same fibre. An alternative possibility is that the same receptor may be commonly stimulated by several different chemicals. In order to investigate this question the technique of selective adaptation was utilized. A single functional "water" fibre was adapted to water by a constant flow of distilled water over the tongue. A solution of 0.009 M quinine hydrochloride in distilled water was then applied to the same receptive field of the tongue and caused a burst of impulses in the associated "water" fibre. A similar application of water following previous water adaptation did not cause any burst of impulses. It should be recalled that when the water response is depressed by previous

application of acid, the response to subsequent acid stimulation is unimpaired. These findings suggest that different kinds of receptors may be attached to the same "water" fibre; one type responds to quinine while the other responds to a flow of water. A less likely possibility is that water and quinine act on different sites of the same receptor. We can not decide between these possibilities with the information available; it can only be concluded that water and quinine initiate impulses in the "water" fibre by different means.

The mechanism of water stimulation. It is not known whether the "water" receptor consists of free nerve endings or whether it is associated with taste buds; for this reason we will use the term "receptor" or "receptor membrane" to mean the structure in which the initial excitatory process is evoked.

The necessity of a flow of water over the receptor surface suggests that removal of ions from the external environment of the receptor membrane is important for stimulation by water. Simple osmotic pressure *per se* is ruled out as a stimulating factor because aqueous isotonic solutions still cause a response. The electrolyte equilibrium may be disturbed by a rapid water flow such that ions are drawn out across the receptor membrane. As suggested by LILJESTRAND and ZOTTERMAN it is this outward ionic flux that appears to initiate the excitatory process. Depression of "water" fibre activity may result from the reciprocal process of ions moving inward across the receptor membrane due to a high external ionic concentration. It seems that the anion is the critical factor in this process. Choline chloride behaved similar to the inorganic salts while high concentrations of organic sodium salts failed to depress the "water" receptor. The failure of organic anions, such as acetate and formate, to depress the "water" receptor may be due to their inability to penetrate the membrane. This further indicates that it is the inward anionic flux that depresses "water" receptor activity. Under physiological conditions the most likely anion involved in water receptor excitability is the chloride ion. Thus in this receptor type it may be that an outward flow of chloride ions facilitates while an inward flow inhibits the excitatory process.

It may be thought that the stimulation of the "salt" and "water" receptors involve mirror image processes such that an inward flow of anions stimulates the former and inhibits the latter. Such is not the case, however: NO^-_3 does not stimulate the "salt" fibre but is as effective as any other inorganic anion in depressing the "wa-

ter" fibre; similarly choline has a specific stimulating effect on the "salt" receptor while no such effect was observed on the "water" receptor. We must conclude, therefore, that excitation in the "salt" and "water" receptors involves fundamentally different mechanisms.

Summary.

1. Taste receptors in the cat's tongue were analyzed by recording from single afferent chorda tympani nerve fibres while applying various sapid solutions to the tongue.

2. Single fibres were observed which responded to the flow of distilled water over the tongue. The activity of these "water" fibres was depressed by various inorganic salt solutions with an anion concentration above 0.03 M. Organic sodium salts, in contrast, stimulated this fibre type in a manner similar to distilled water.

3. The "water" fibres were also stimulated by quinine hydrochloride and mineral acids below pH 2.5.

4. Single "salt" fibres were observed which responded to various salts above 0.1 M concentration. These fibres were also stimulated by acid.

5. Another fibre type responded primarily to quinine chloride and very little if at all to strong acid. The "quinine" fibre was not stimulated by hypertonic salt solutions or by distilled water.

6. The fourth fibre type found responded only to acids below pH 2.5.

7. It is proposed that the presence of the "water" fibre extends the sensitivity to salt solutions beyond the concentration limits served by the "salt" fibres thus enabling discrimination of salt concentration over the entire physiologically significant saline range.

8. The importance of impulse pattern is discussed and a scheme presented illustrating the possible relationship between afferent impulse pattern and the evoked taste sensation.

9. The possible mechanism of stimulation by water is discussed and a view presented that the outward flow of anions across the receptor membrane stimulates while an inward flow depresses "water" fibre activity.

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From the Department of Physiology, Kungl. Veterinärhögskolan, Stockholm.

A Further Study of Polydipsia Evoked by Hypothalamic Stimulation in the Goat.

By

BENGT ANDERSSON and S. M. McCANN.

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It has been reported earlier that hypertonic sodium chloride solutions could produce primary polydipsia when injected into a certain region of the hypothalamus of the goat (ANDERSSON 1952, 1953). The injection of about 0.1 ml of a 1.5 to 2 % sodium chloride solution into the medial hypothalamus induced the animals to drink large volumes of water in many cases. In order if possible to obtain a more precise localization of the portion of the hypothalamus concerned with this response, microinjections, as reported in the following, were performed. Since it has been impossible to determine the effective spread of these injections and the effect obtained often has been difficult to repeat, the study has also been extended to include electrical stimulation.

Methods.

Full grown, female goats were used for the experiments. Microinjections into the hypothalamus were performed according to a method described elsewhere (ANDERSSON and LARSSON 1955). For electrical stimulation, HESS' technique (1932, 1949), somewhat modified for use in goats (ANDERSSON 1951) was used. Three electrodes could be stimulated, either simultaneously or individually, at a given depth in the hypothalamus with this technique.

Parts of the experiments were filmed. The films are available on request.

To obtain precise records of urine flow, the animals were occasionally placed in a modified Pavlov stand, and the diuresis was recorded with the aid of a "Dowse's" selfretaining catheter.

Blood samples were taken from the jugular veins. The hematocrit value was determined by centrifugation of 10 ml samples in graduated tubes at 4,000 r. p. m. for 20 minutes, using heparinized blood. Blood glucose was determined by the method of FOLIN and WU (1930).

Sodium and potassium analyses in plasma and urine were made using the EEL flame photometer. Plasma chloride determinations were made according to the silver nitrate method of VAN SLYKE (1923), and urine chloride according to the Volhard method (PETERS and VAN SLYKE 1932).

Histological technique. After killing the goats by decapitation, the heads were perfused with Bouin's fixative. After fixation in Bouin's and paraffin imbedding, the hypothalamus with attached pituitary was serially sectioned and stained by Gomori's chrom alum hematoxylin method (GOMORI 1941) as described by BARGMANN (1950). The localization of the effective points of stimulation was in most cases facilitated by the injection of minute amounts of 1 % osmium acid or by electrocoagulation just prior to killing the animals.

Results.

A. *Microinjections into the hypothalamus.*

Injections of 0.003 to 0.01 ml of 2 to 3 % sodium chloride solutions into different parts of the hypothalamus were made in 16 experiments. The injection technique made it possible to inject the solutions at several locations in each experiment. The area of the hypothalamus where injections of this kind had an obvious polydipsic effect was more restricted than that found when using larger amounts of hypertonic saline (ANDERSSON 1953). This area is projected on Figures 1 and 2. Microinjections into other regions of the hypothalamus failed to produce polydipsia, however, no systematic exploration has been made of the region just dorsal to that which induced drinking.

Positive results were obtained from one or more points of stimulation in 8 of these experiments. The volume of water drunk as a consequence of such injections varied between 2 to 8 liters. The time of onset of drinking varied between 30 to 60 seconds after the injection, and the goats drank more or less continuously for two to five minutes thereafter. The effect was, however, not always repeatable on renewed injection. It could be obtained three times at the most. Other effects such as increased

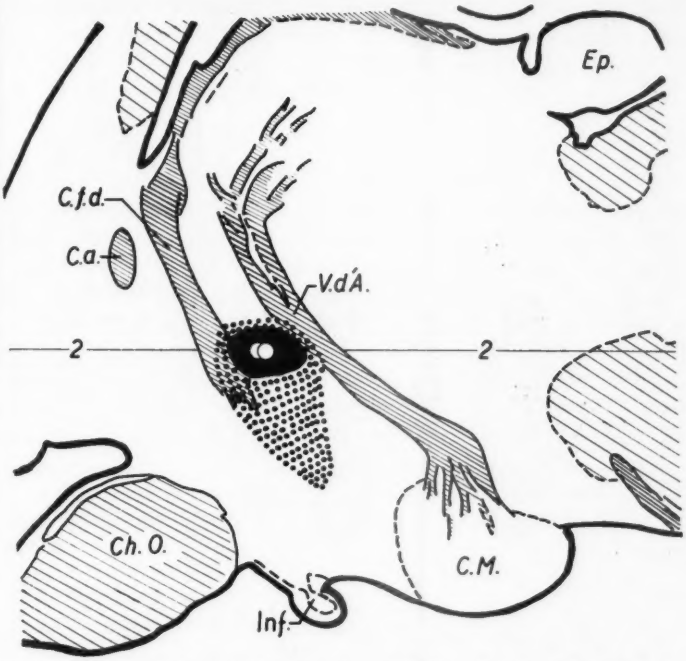

Fig. 1. A diagram of a sagittal section through the hypothalamus of the goat at the level of Columna fornicis descendens and Tractus Vicq d'Azyr.

The dotted area represents the region of the hypothalamus where microinjections of hypertonic NaCl solutions had a polydipsic effect.

The black area represents the locus where electrical stimulations caused drinking. (The white circles within this area mark the points where the most obvious polydipsic effect was obtained by electrical stimulation; exp. I and II in the text.)

The level of a horizontal section shown in Fig. 2 is indicated by the line labelled 2.

C. a.: Commisura anterior.
C. f. d.: Columna fornicis descendens.
C. M.: Corpus mammillare.
Ch. O.: Chiasma opticum.
Ep.: Epiphysis cerebri.
Inf.: Infundibulum.
V. d'A.: Tractus Vicq d'Azyr.

food intake, licking, etc., were also seen in some cases following the injections, but in many cases there were no observable effects other than drinking.

The most obvious polydipsia was obtained in two experiments. These two goats drank 6 and 8 liters of water, respectively, as a result of the injections. Especially if the stimulus was repeated

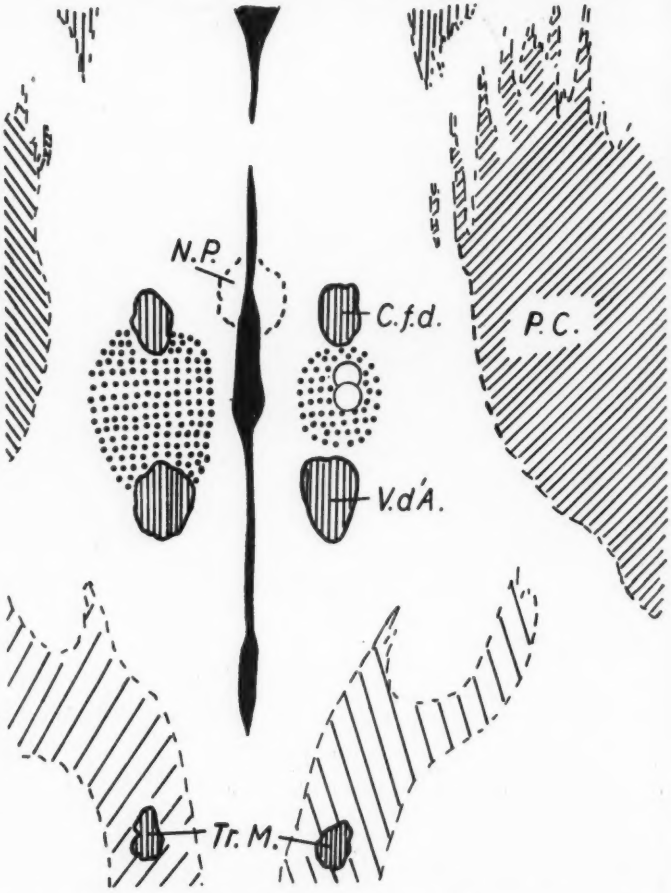

Fig. 2. A diagram of a horizontal section of the hypothalamus of the goat at the level indicated by the line labelled 2 in Fig. 1.

The dotted area on the left side represents the region where microinjections of hypertonic NaCl solutions produced polydipsia.

The dotted area on the right side represents the area where electrical stimulation caused drinking. (The white circles within this area mark the points where the most obvious polydipsic effect was obtained by electrical stimulation; exp. I and II in the text.)

C. f. d.: Columna fornicis descendens.
P. C.: Pedunculus cerebri.
V. d'A.: Tractus Vicq d'Azyr.
Tr. M.: Tractus Meynert.
N. P.: Nucleus Paraventricularis.

after such overdrinking, the subsequent water diuresis was more delayed in onset and associated with greater hemodilution than seems to be the case when equivalent amounts of water are administered to goats by ruminal fistula (ANDERSSON 1955).

B. *Electrical stimulation of the hypothalamus.*

This type of stimulation was carried out in 7 experiments on 6 goats with the aim to study the water intake. In each experiment, many points in the hypothalamus were stimulated. Polydipsia was obtained from 5 different points of stimulation. These points were found to lie within a strictly limited area between Columna fornicis descendens and Tractus Vicq d'Azyr, at the horizontal level of the middle of the hypothalamus (Figs. 1 and 2). The most striking polydipsic effects were obtained in two goats. The points of stimulation in these two cases were immediately adjacent to each other. Their location is shown in Figs. 1 and 2.

The following abbreviated protocols of these two experiments document more fully the remarkable reproducibility of the phenomenon.

I. Dec. 3, 1954 Goat "G". Weight: 39 kg.

Time:

11.45 Operation completed, animal in good condition.

12.00—15.00 Stimulations 1 to 2 mm from the point which later gave the most pronounced drinking induced drinking of approximately 3 liters of water. Since stimulation was not always effective, the electrodes were then moved slightly.

15.03—15.25 The animal drank during each of 6 consecutive stimulations by the most rostral of the three electrodes (stimulus strength 0.5—1.0 Volt, 50 cycles/sec.). The time of latency before the onset of drinking varied between 9 and 25 seconds. The stimulations were continued for 10 to 20 seconds after drinking had started. The goat stopped drinking 2 to 3 seconds after cessation of stimulation. Five liters of water were consumed during the 6 stimulations.

15.27 After 2 more effective stimulations, the stimulus was repeated as above, but the animal was now offered a mixture of urine and water. She started to drink after 10 seconds and continued to drink steadily during the 8 seconds the stimulation was continued and for 2 seconds thereafter. By this time the animal had drunk an additional 2 liters of water.

15.45 After another positive stimulation at this point, she was again stimulated for 4 minutes in an attempt to produce extreme overhydration. During this period she drank with occasional short interruptions another 4.5 liters.

16.00 Blood sample: Hematocrit 31, blood glucose 77 mg/100 ml, plasma sodium 145 meq./l, plasma chloride 97 meq./l, slight hemolysis was noted in the plasma.

16.40 After two brief effective stimulations by the rostral electrode, a slight effect was obtained by the middle electrode, whereas stimulation by the caudal electrode at the same strength was completely ineffective. The animal now looked sick and exhibited rapid, shallow breathing. The goat had now drunk 16 liters during the whole course of this experiment. Her rumen was quite hard and distended by water (Fig. 3, I a). Large volumes of dilute urine were voided containing considerable hemoglobin. Blood sample: Hematocrit: 29.7, blood glucose: 84 mg/100 ml, plasma sodium 140 meq./l, plasma chloride 94 meq./l, marked hemolysis.

16.53 Electrocoagulation was performed at the effective point of stimulation. The electrodes were then removed, and the animal was left overnight with free access to food and water.

Dec. 4, 9.20 The animal appeared to be in good condition, having taken no water overnight (Fig. 3, I b). A marked water diuresis was still in progress, and the urine contained considerable hemoglobin. It had a specific gravity of 1.002.

Blood sample: Hematocrit 25, blood glucose 79 mg/100 ml, plasma sodium 132 meq./l, plasma chloride 59 meq./l.

11.00 The goat was killed; at autopsy the rumen still contained a considerable volume of fluid.

II. Nov. 16, 1954 Goat "E". Weight: 40 kg.

11.20 Operation finished. For the volume of water drunk, the blood samples taken, and the urine voided during the first part of this experiment, see Table 1.

11.30—15.30 At two points on the left side of the hypothalamus, stimulation induced drinking of water, but the effect in one case was not repeatable and in the second could only be repeated once. Repeated stimulation at 6 points in the ventral hypothalamus at the same sagittal level on this and the opposite side of the brain failed to elicit drinking.

15.30 Stimulation was performed at a new level more dorsal in the hypothalamus on the right side by all three electrodes simultaneously (1.5 Volt, 50 cycles/sec.) for 5 minutes. After about 30 seconds of stimulation, the animal began to drink and drank almost continuously until the stimulus was discontinued.

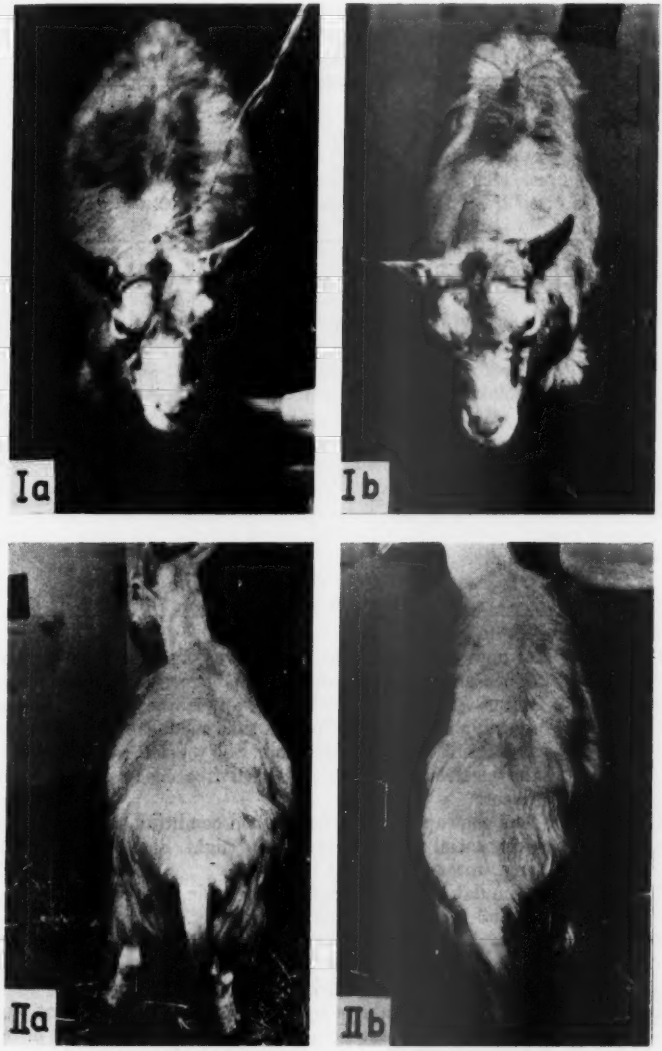

Fig. 3. This figure contrasts the appearance of two goats (exp. I and II in text) after overhydration induced by electrical stimulation to their appearance 15 hours later when most of the water had been excreted.

Table 1.

The amount of water drunk and the time course of the hemodilution and water diuresis induced by electrical stimulation in the hypothalamus during the first part of exp. II. (See text.)

Time	Liters of water drunk	Blood					Urine	
		Hematocrit	Glucose mg/100 ml	Plasma meq./l			Volume in ml	Spec. gravity
				Na	K	Cl		
1954								
Nov. 16th								
11.20		33	79	146	4.5	106		
11.20—13.01	1						130	1.020
13.01—14.17							108	1.025
14.25—14.45	3½							
15.30—15.35	6							
14.17—15.40							87	1.025
16.25		31	67	141	4.0	100		
15.40—16.42							190	1.015
17.04		29	69	139	4.2	97		
16.42—17.05							250	1.003
17.14	1							
17.05—17.44							388	1.001
Nov. 17th								
9.00		31	76	148	4.5	114		

17.14 A new stimulation as above was again effective. The appearance of the goat at this time, overloaded with water, is compared with its appearance the following morning in Fig. 3, II. By this time she looked ill, probably as a result of the overdrinking. She was left overnight with the electrodes in situ and with free access to food and water.

Nov. 17,9.00 The goat appeared to be in good condition and had eaten substantial amounts during the night. She had not drunk any water (Fig. 3, II b).

10.10 Stimulation by the rostral electrode, strength as above, for 90 seconds induced no drinking.

10.13 Stimulation as above at the caudal electrode was effective, and the animal drank 1 liter, until the stimulation was discontinued, when drinking ceased.

10.15—10.29 Stimulation as above by the caudal electrode was carried out on 6 different occasions. After a short period of latency, varying between 10 and 30 seconds, the goat drank in all cases as long as the stimulus was continued. She continued to drink on the average for 3 to 4 seconds after the stimulation was discontinued.

10.40 Stimulation by the rostral electrode as above for 90 seconds induced no drinking.

10.43 A stimulus delivered by the caudal electrode was again effective.

10.45 On stimulating by the middle electrode for 90 seconds as above, the animal lowered her head to the water several times as if to drink but failed to do so.

10.48 Stimulation by the caudal electrode was again effective after 12 seconds latency. During the stimulations this morning, the goat had now consumed 5 liters of water.

11.00 She was now stimulated by the caudal electrode as previously and consumed during a period of $2^1/_2$ minutes of stimulation 6 liters of water. She drank until cessation of stimulation.

To study the eventual effect of stimulating at the effective point on the water diuresis produced by the 11 liters of water she had now drunk, the animal was placed in a modified Pavlov stand with retention catheter in the bladder. No further stimulations were carried out for more than 2 hours.

13.11 Stimulation was performed by the caudal electrode for 5 minutes as described above. The goat drank water when it was offered 30 seconds after onset of stimulation and still wanted it just before the stimulation ceased. She was, however, only allowed to drink negligible amounts during this period. The stimulation caused a marked inhibition of her water diuresis and increased the urinary concentration of electrolytes without altering the total electrolyte excretion (Fig. 4).

15.25 Repeated stimulation by the caudal electrode once again induced drinking. During all stimulations at this most effective point, the goat was quite calm. The only associated effect was occasional licking. The location of this point of stimulation is portrayed on Figs. 1 and 2.

In general, the results from electrical stimulation have shown that in the goat this is a convenient, effective way to study hypothalamic polydipsia. The area concerned in this response could be more closely localized in this way (Figs. 1 and 2). The experimentally induced drinking from electrical stimulation could be repeated at will. The drinking was clearly related to the stimulation since it commenced 10 to 30 seconds after the onset of the stimulus and continued for only 2 to 3 seconds after the current was discontinued. Drinking once initiated characteristically continued throughout the period of stimulation. It was very forceful in character and even diluted urine could be taken. Marked overhydration up to 40 % of the body weight could result from these stimu-

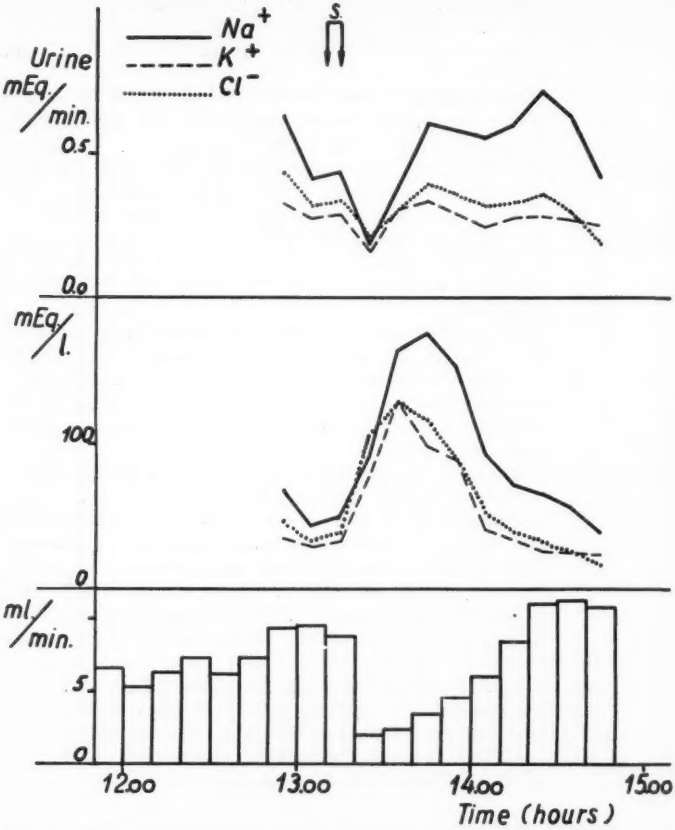

Fig. 4. The effect of an electrical stimulation producing polydipsia on the urine flow and urinary concentrations and excretion of sodium, potassium and chloride in exp. II. (Stimulation: 1.5 Volts, 50 cycles/sec. for 5 minutes.) No corrections have been made for dead space in the urinary tract. This probably accounts for the transient decrease in electrolyte excretion at the onset of the inhibition of the water diuresis.

lations, accompanied by subsequent hemodilution and profuse diuresis. The hemodilution appeared to be more marked and the diuresis more delayed than that from administration of equivalent amounts of water through a ruminal fistula (ANDERSSON 1955). Stimulation at the same point where polydipsia was induced produced a marked antidiuresis with a similar time course to that

following the intravenous injection of posterior pituitary extract to goats (Fig. 4) (ANDERSSON 1955). This antidiuresis was not accompanied by any significant change in urinary electrolyte excretion.

Drinking from electrical stimulation could occur in the absence of other demonstrable effects, but was associated at times with licking, exploratory activity, or turning movements. Similar effects were obtained from other regions of the hypothalamus in the absence of drinking.

Discussion.

Several hypotheses have been proposed to explain the sensation of thirst. Much evidence indicates that CANNON's theory (1918) that the sensation arises from the mouth inadequately explains the feeling of thirst (BELLOWS and VAN WAGENEN 1939, STEGGERDA 1939). According to another widely held theory, the sensation is closely correlated with cellular dehydration (GILMAN 1937). WOLF (1950) on the basis of his osmometric analysis of thirst has even postulated that "osmoreceptors" such as those which probably govern the secretion of antidiuretic hormone (VERNEY 1947) might also regulate water intake.

Evidence for the view that the hypothalamus might play a rôle in the regulation of water intake was provided by the observation that primary polydipsia could be evoked by injections of hypertonic saline solutions into the hypothalamus of the goat (ANDERSSON 1952, 1953). The choice of a ruminant animal such as the goat for experiments of this type appears to offer several advantages. The voluminous rumen can function as a fluid reservoir (ANDERSSON 1955), which permits consumption of large volumes of water before significant inhibitory influences from the distension of the digestive tract seem to suppress the thirst mechanism. Furthermore, the placid goat is very well suited for hypothalamic stimulation in the conscious state.

The microinjections reported here have more closely localized the region of the hypothalamus where stimulation of this kind can evoke drinking. As, however, other effects were sometimes seen in association with polydipsia from the injections, conclusive proof has not been obtained for the view that the osmotic stimulus was the adequate stimulus to the tissue producing the drinking

response. But that the osmotic stimuli may preferentially excite the "thirst mechanism" is indicated by the fact that electrical stimulation of the regions adjacent to the most effective area usually gave more side effects than similarly placed injections.

Insufficient reproducibility and the failure to obtain a more exact localisation appeared to constitute the principal disadvantages of this type of stimulation. Both of these disadvantages have been circumvented by electrical stimulation of the hypothalamus. When stimulating electrically, it was possible to repeat the effect very frequently and without fail. The fact that during this type of stimulation the animal drank a urine water mixture indicates that the urge to drink was very strong. Furthermore, the short time of latency before the onset of drinking and the short period of drinking after the cessation of stimulation clearly relates the polydipsia to the applied stimulus. The fact that stimulation continued over longer periods, when the animals where already considerably hydrated, could produce an enormous overhydration shows that any inhibitory mechanism in these cases was insufficient to overcome the experimentally induced drinking. This taken together with the marked hemodilution found here clearly proves the primacy of the polydipsia from these hypothalamic stimulations.

Especially when repeated stimulations were applied after overhydration the subsequent water diuresis seemed to be delayed in its onset and the decrease in plasma sodium and chloride values was more marked than that previously seen during overhydration through a ruminal fistula (ANDERSSON 1955). To further elucidate this phenomenon the effect of stimulation on the water diuresis was evaluated as described above. In this goat an inhibition of water diuresis which was clearly of neurohypophyseal type resulted from stimulation of the same area which induced drinking. Further work is necessary to determine if this would always be the case. The result could, however, be taken as support for a view that a hypothalamic mechanism regulating the water intake also exerts an influence on the secretion of antidiuretic hormone from the neurohypophysis. If the inhibition was due to antidiuretic hormone, as indicated by its onset and time course, the determination of urinary electrolytes shows that the endogenously released hormone under these conditions had no obvious natriuretic or chloruretic effect (Fig. 4).

The active region of the hypothalamus determined by microinjections and electrical stimulation is included within the somewhat

larger area which was previously found using larger volumes of hypertonic saline (ANDERSSON 1953). The portion of the middle hypothalamus which appears to be concerned with water intake is included in the area where HESS in his stimulations, compiled by BRÜGGER (1943), found bulimia in cats. In some of these experiments, the cats showed a preference for milk as contrasted to meat, suggesting that they might also have drunk water if it had been offered to them. The polydipsia seen in the experiments reported here has been completely separated from hyperphagia, which would be consistent with the idea that the region governing food intake may lie more laterally (ANAND and BROBECK 1951, LARSSON 1954).

Summary.

1. Microinjections of 0.003 to 0.01 ml of 2 to 3 % sodium chloride solution into a fairly limited part of the hypothalamus of the goat produced drinking of from 2 to 8 liters of water on one or more occasions in 8 experiments in 16 goats.

2. Drinking commenced 30 to 60 seconds after the injection and continued for 2 to 5 minutes.

3. The polydipsic effect of the injections was not always repeatable.

4. Electrical stimulation of the hypothalamus in 7 experiments produced drinking from 5 different points of stimulation.

5. In contrast to the injection experiments, polydipsia could be produced at will by electrical stimulation of a discrete region of the hypothalamus lying between Columna fornicis descendens and Tractus Vicq d'Azyr.

6. Drinking caused by electrical stimulation commenced 10 to 30 seconds from the onset of stimulation and stopped 2 to 3 seconds after the current was discontinued.

7. Tremendous overhydration could be induced in this way followed by marked hemodilution and polyuria.

8. When tested in one experiment, the stimulation at the same point, where polydipsia was evoked, produced a marked antidiuresis of neurohypophyseal type.

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From the Zoophysiological Laboratory, University of Copenhagen and the Institute of Hygiene, University of Aarhus.

Studies on the Active Transport of Lithium in the Isolated Frog Skin.

By

K. ZERAHN.

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In biological systems the behavior of lithium is often more similar to that of sodium than to that of any other ion. It was therefore of interest to determine how nearly lithium could replace sodium in the active transport system of the isolated surviving frog skin.

Investigations on this organ with respect to lithium have been previously performed by GALEOTTI (1904) who found that the isolated frog skin will maintain approximately the same potential difference of 50—100 mV when immersed in 0.1 molar lithium chloride as when bathed in 0.1 molar sodium chloride.

The transport of lithium by the isolated surviving frog skin was investigated by the technique of short-circuiting the skin. USSING and ZERAHN (1951) and H. LINDERHOLM (1952) have demonstrated that in the isolated frog skin immersed in Ringer's solution, the short-circuit current is equal to the net sodium flux (Δ Na) through the skin. This technique has been further applied in the work of KOEFOED-JOHNSEN, USSING and ZERAHN (1952). With this background it seemed appropriate to apply the same technique to the study of the active transport of lithium.

Methods.

The technique and apparatus described by USSING and ZERAHN (1951) as modified by KOEFOED-JOHNSEN, USSING and ZERAHN (1952) were used.

Three different types of experiments were included in this study:

A. *The addition of known amounts of lithium to the Ringer's solutions on both sides of the frog skin.*

Influx and outflux of sodium through the frog skin were determined by the double labelling method (LEVI & USSING 1949), using the two radioactive isotopes Na^{24} and Na^{22}. Control flux measurements were obtained with Ringer's solution bathing both sides of the skin. Then varying amounts of lithium were added to the bathing solutions, keeping the concentrations of lithium the same on both sides of the skin. The concentrations of lithium were obtained by replacing the Ringer solution bathing the skin during the control periods with known volumes of a Ringer solution in which all of the sodium has been replaced by lithium (Li-Ringer).

The use of agar-Ringer bridges in these experiments does not introduce a significant error in the measurements.

B. *The replacement of sodium in the solution bathing the outside surface of the skin by an "inert" ion.*

The skin was mounted in the usual fashion with Ringer solution bathing both surfaces. The skin potential was observed until nearly constant values were obtained. The short-circuit-current was then measured and the outside solution was replaced with a magnesium Ringer solution (Mg-Ringer) in which an equivalent concentration of magnesium replaced all the sodium. The outside surface of the skin was rinsed 2—3 times with this solution and the outside Ringer-agar bridge was replaced by a Mg-Ringer-agar bridge. Substitution of Mg for all of the sodium in the outside solution stops the active transport and abolishes the usual short-circuit current, as Mg is unable to replace Na in the transport system. The skin potential becomes slightly negative and the direction of the short-circuit current is reversed. The small, reversed short-circuit current of 0.3—5 μAmp./cm^2 is attributable to the outward diffusion of Na through the skin. Either Na-Ringer or Li-Ringer, or a mixture of the two, could now be added to the outside and the effects on the short-circuit current determined. That the skin preserved its normal transport mechanism in the presence of the high Mg-concentrations was indicated by the good agreement between Δ Na and the short-circuit current when Na was added to the outside solution. Though the Mg-Ringer outside has a lower osmolarity than the Ringer inside, the resulting slow flow of water inward through the skin did not interfere with the experiments.

In these experiments the sodium flux was measured by the double labelling technique.[1] The lithium influx alone was measured, as the inside solution initially contains no lithium. The lithium influx was determined as the sum of the quantity which entered the inside solu-

[1] The Na^{24} used in these experiments was prepared by Philips Roxane Isotope Laboratories, Amsterdam, by bombardment of aluminium with deuterons and purified to less than 1 ppm of radioactive impurity remaining after 14 days.

tion plus the lithium present in the skin. This latter quantity of lithium was considered to have participated in the active transport process. That this moiety of lithium in the skin did not represent merely solution adsorbed on the skin was shown by estimating separately this latter quantity of solution. In these experiments the calcium in the outer solution of Mg-Ringer was labelled with Ca^{45}. As it was found that the calcium does not disappear appreciably from the outer solution even over a period of several hours, the quantity of adsorbed calcium was measured and taken as a measure, undoubtedly a maximal value, of the quantity of solution adhering to the skin. It was found that this quantity was very small, less than 10 μl of solution, and the lithium content of the skin has been appropriately corrected for adhering solution. After the outside of the skin has been in contact with the Li-Ringer solution for the desired time, the applied short-circuit voltage was disconnected, the skin potential measured and the bathing solutions removed. The apparatus was then quickly taken apart. With the outside of the skin adhering to the rim of the chamber, the inside surface was wiped with filter paper and the free circular part of the skin was cut out.

During this operation the skin was supported on filter paper. When cut free it was dried with filter paper, weighed, and ashed at 500° C. The ash was dissolved in 2.0 ml of 0.1 N hydrochloric acid and the solution was transferred to a centrifuge tube, the quartz crucible being washed twice with 2 ml portions of water. The calcium was precipitated as calcium oxalate and the precipitate was transferred directly to aluminium dishes for measurement of the radioactivity of the labelled calcium. The calcium specimens were counted against a standard obtained from the corresponding solution bathing the outside of the skin. The total Ca^{45} counts of the skin compared with the concentration of Ca^{45} in the outside solution then gives an estimate of the volume of outside solution adhering to the skin. The quantity of lithium in this volume of solution was subtracted from the total lithium content of the skin[1] to yield that quantity of lithium in the skin which had participated in the active transport process. The radioactivity of Ca^{45} was so low that it did not disturb the measurements of the Na^{24} samples from the outside because the β particles from these samples were measured through 0.5 mm of aluminium. The samples of Na^{22} from the inside did not contain significant amounts of Ca^{45}. The lithium content of the Ringer solution bathing the inside of the skin was measured with a Beckman flame photometer. Because of the high concentrations

[1] In determining the Li content of the skin with the Beckman flame photometer it was necessary that the Ca be removed from the solution because the wide slit used in determining the lithium does not cut out all the light from the 622 mμ band of Ca. It was necessary to use a wide slit of 0.2 mm because of the very low concentrations of Li.

In the frog Rana temporaria, the amount of Ca is always very small — about one millimol for a 7 cm^2 skin — while in the frog Rana esculenta, the same area of skin will have about 30 millimol of calcium. Thus in the latter instance the high calcium concentrations can be the source of a rather large error. Therefore, all lithium determinations were made on solutions free of calcium.

of sodium in the inside solution, circa 115 mEq. per liter, the standard lithium solutions were prepared by diluting known amounts of Li-Ringer to volume with Na-Ringer. In addition Na-Ringer was used as a blank.

C. *Demonstration of the transport of lithium against an electrochemical gradient.*

If the movement of lithium through the frog skin is the result of an active transport process it should be possible for the lithium ions to be moved against an electrochemical gradient. In the previous experiments conditions were such that the concentration gradient for lithium was either zero, as in Part A, or positive, as in Part B with lithium only added to the outside bathing solution. In order to obtain a measurable change in lithium concentration resulting from its transport through the skin, it was desirable to work with smaller volumes of solution than required in the apparatus for measuring the "short-circuit" current. Thus these experiments were performed with the skin bag technique used by HUF (1935). The skin from the hind legs of Rana esculenta was carefully dissected free and a bag made by tying off one end with a thread. The open end was tied to a glass tube. A cork stopper with a slit was placed in the glass tube and the bag was dipped into a beaker containing 30 ml of an aerated solution.

This solution consisted of 2.20 mM per liter of lithium. The skin bag was filled with 2 ml of a solution of known amounts of Li-Ringer added to ordinary Ringer solution after first being rinsed three or four times with this solution. This refers to the two first experiments in Table 5; in the following experiments the epithelial side was turned inward, and the solutions were changed accordingly. After the skin bag was filled, it was quickly weighed and placed in the beaker, with care taken to adjust the height of the bag so that inside and outside fluids were at the same level. During the experiment frequent measurements of the potential difference across the skin were made. At the termination of an experiment the bag was weighed again to obtain the change in volume of inside solution. The sign for both potential difference and Δ V refers to the chorion side. Finally the lithium content of the bag was determined by flame photometry.

Results.

Figure 1 shows the effects on the short-circuit current of adding different concentrations of lithium to the solutions bathing both sides of the frog skin. High concentrations of lithium on both sides definitely depress the current. Concentrations of 20 % or less do not have much effect.

Table 1 shows the results of a series of experiments in which varying amounts of Li-Ringer were added to both sides of the skin (see Part A of Methods). It is evident that the short circuit current is equal to the net sodium flux (Δ Na) in all four con-

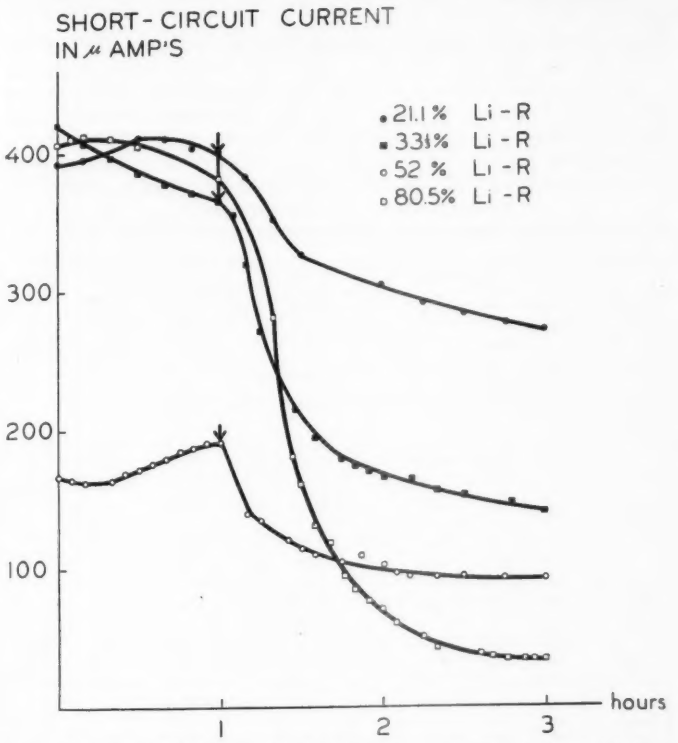

Fig. 1. The effect of Li ions on the short-circuit current of the isolated skin from Rana temporaria. Li was added at the arrow in equal amounts to both sides of the skin. It is seen, that the current is decreased in all cases, but it should be kept in mind that the general trend without Li will be a slow decay of the current with time.

trol periods. After the addition of the Li-Ringer, however, the net sodium transport falls in every instance significantly below the value of the short circuit current. This indicates the presence of an "extra current" in excess of the current which can be attributed to active transport of sodium ions. Furthermore the ratio of this "extra current" to the total short circuit current (expressed in % in the last column) is approximately equal to the ratio of lithium to sodium plus lithium concentrations (third column) in the bathing solutions. This agreement between the "extra current" and the lithium concentration suggests that the former

Table 1.

Results from double labelling experiments with Na^{22} and Na^{24} on the short-circuited frog skin. The first period is a control period, at the start of the following period the Li was added in the form of Li-Ringer, on both sides of the skin, in such amounts that the solutions on both sides of the skin were identical. In column 3 are given the ratio of the concentration of Li and Na+Li, in column 10 the ratio of extra current and total short-circuit current times 100. All experiments were performed on isolated abdominal skins from Rana temporaria.

Date	Time in hours	% Li-R in solution	Influx μEq Na h^{-1} cm^{-2}	Outflux μEq. Na h^{-1} cm^{-2}	Δ Na μEq. Na h^{-1} cm^{-2}	Δ Na μAmp. cm^{-2}	Short circuit current μAmp. cm^{-2}	Extra current μAmp. cm^{-2}	Extra current in % of short-circuit-current
October 14	1	0	2.30	0.13	2.17	58.2	57.0		
	1	21.2	1.20	0.10	1.10	29.5	40.2	10.7	26.6
	1	21.2	0.99	0.09	0.90	24.1	35.3	11.2	31.7
October 15	1	0	2.14	0.09	2.05	54.9	54.8	—	—
	1/4								
	1	33.3	0.85	0.06	0.79	21.2	27.5	6.3	22.9
	2	33.3	0.52	0.07	0.45	12.1	18.6	6.5	34.9
October 1	1	0	0.96	0.14	0.82	22.0	24.5	—	—
	1/4								
	65/60	52.0	0.40	0.06	0.34	9.1	15.2	6.1	40.1
	1	52.0	0.31	0.10	0.21	5.6	12.9	7.3	56.6
October 2	1	0	2.39	0.18	2.21	59.2	56.7	—	—
	1/2								
	1/2	80.5	0.23	0.04	0.19	5.1	14.7	9.6	65.3
	1	80.5	0.12	0.05	0.07	1.9	6.2	4.3	69.4

results from the active transport of lithium and that the participation of lithium in the active transport system is proportional to its mole fraction of the sodium and lithium present in the bathing solution.

In Table 2 are shown the results of experiments in which known amounts of sodium and/or lithium have been added to the inert Mg-Ringer solution bathing the outside of the skin (see Part B of Methods). As seen in the first period of the experiment of April 1, when Mg-Ringer solution alone is in contact with the outside of the skin the electrical activity of the skin is minimal (short circuit current is negative) and leakage of sodium occurs outward through the skin. When either sodium ions and/or

Table 2.

Results from experiments with magnesium-Ringer on the outside of the isolated abdominal skin from Rana temporaria. The amounts of sodium passing in and out through a 7.06 cm² skin are measured with Na^{22} and Na^{24} and the amounts of Li passing in by analysis of the inside solution and of the content in the skin. Due corrections are made for the outside solution adhering to the skin. The contribution of Na and Li ions correspond fairly closely to the amount of electricity used for short-circuiting the skin. All other ions involved are in the same concentration on the inside and on the outside, except the magnesium.

Date	Time in hours	Concentr. outside µmol/ml		µmol of Na passing		µmol of Li		Net amount ions in µmol	Electrical amount of shorting current in µEq.
		Na	Li	in	out	in skin	inside		
Feb. 10	2½	9.7	9.7	3.28	1.41	0.68	1.88	4.43	4.78
Feb. 11	1	0	11.0	0	0.77	0.73	1.33	1.29	1.45
March 17	1	2.8	0	2.21	0.65	0	0	1.56	1.53
I	1¾	2.7	2.8	1.88	1.19	0.51	0.76	1.96	1.95
March 17	1	2.8	0	2.58	0.63	0	0	1.95	2.02
II	1½	2.7	5.8	1.58	0.87	0.54	1.38	2.63	2.41
April 8	1	0	0	0	0.41	0	0	−0.41	−0.07
	1¼	3.1	0	1.93	0.45	0	0	1.48	1.50
	1½	2.9	6.4	1.53	0.62	0.84	1.60	3.35	3.53

lithium ions are added to the outside solution, however, as in all the other periods shown, the positive value of the short circuit current is reestablished. The net movement of lithium plus sodium ions through the skin was determined in these experiments as the difference between sodium influx and outflux plus the sum of the lithium transported to the inside solution and the lithium present in the skin (see Discussion). The good agreement between the value for the net amount of ions transported and the short circuit current, whether the ions involved were sodium alone, lithium alone or a combination of the two, establishes that the short circuit current is accounted for solely by the movement of lithium and sodium ions.

Table 3.

Results from experiments with magnesium Ringer on the outside of isolated skins from Rana esculenta. Experimental conditions as in Table 2.

Date	Time in hours	Concentr. outside μmol/ml		μmol of Na passing		μmol of Li		Net amount ions in μmol	Electrical amount of shorting current in μEq.
		Na	Li	in	out	in skin	inside		
March 26	1	2.8	0	1.25	1.34	0	0	−0.09	0.22
	1½	2.8	3.0	1.22	1.93	0.59	0.41	0.29	0.34
March 26	1	10.4	0	3.35	0.64	0	0	2.71	2.35
	2	9.5	10.2	3.16	1.53	1.23	1.64	4.50	4.56
March 27	1	0	0	0	0.56	0	0	−0.56	−0.15
	1½	0	3.1	0	1.17	0.65	1.14	0.62	1.18
March 28	1	0	0	0	0.20	0	0	−0.20	−0.93
	1½	0	6.1	0	0.85	1.13	3.40	3.68	3.75

Table 3 shows the results of similar experiments performed on a different species of frog, Rana esculenta. The findings are in accord with those presented in the preceding table.

In Table 4 are shown the results of additional similar experiments in which choline, rather than magnesium, was the "inert ion" in the outside bathing solution. Again the results are similar, though the measured net amount of ions transported is significantly less than the short circuit current in all but one period. This discrepancy averaged 1.2 μEq. per hour and was larger than that expected from the experimental error. This suggests that the choline may possibly stimulate the skin glands or may itself penetrate the skin sufficiently to contribute to the short circuit current. The large concentration gradient for choline inward across the skin would favor such movement. However no penetration of the frog skin by choline could be demonstrated by Dr. L. KIRSCHNER.[1] In any case choline would seem to be a less satisfactory "inert ion" than magnesium in these experiments.

Further similar experiments were made using calcium as the "inert" ion on the outside. In 13 periods with the total duration

[1] Personal communication.

Table 4.

*Results from experiments with choline-Ringer on the outside of the isolated skin from Rana temporaria and Rana arvalis.**
The amounts of sodium passing in and out are measured with Na^{22} and Na^{24} respectively and the amount of lithium passing in by analysis of the inside solution and the skin. Due corrections are made for the outside solution sticking to the skin. It seems that there is some ion movement which is not accounted for by the Na and Li.

Date	Time in hours	Concentration outside µmol/ml		µmol of Na passing		µmol of Li		Net amount of ions in µmol	Shorting current × time µEq.
		Na	Li	in	out	in skin	inside		
*April 9	1	0	0	0	1.07	0	0	−1.07	0.20
	1	0	0	0	0.78	0	0	−0.78	0.24
	1	3.3	0	2.32	0.69	0	0	1.63	2.24
	1½	3.1	6.8	2.91	1.37	0.93	1.62	4.09	4.86
*April 30	1	0	0	0	1.12	0	0	−1.12	0.52
	1	3.1	0	5.56	1.25	0	0	4.31	5.08
	1	2.9	6.4	3.05	1.53	0.86	1.72	4.10	5.00
*May 15	1	0	0	0	0.55	0	0	−0.55	−0.48
	1	0	5.0	0	1.37	0.73	1.50	0.86	0.60
May 15	1	0	0	0	3.31	0	0	−3.31	−0.08
	1	4.8	0	5.70	2.70	0	0	3.00	4.8
	1½	4.1	17.0	2.20	3.50	1.36	4.02	4.08	7.8

of 16 ¾ hours the current was found to exceed the net flux of sodium + lithium by an average value of 0.36 µEq./h. When the penetration of calcium through the skin was measured in two 2 hour periods using Ca^{45} as a tracer an average rate of penetration of 0.31 µEq. per hour was found. This indicates that penetration of calcium satisfactorily accounts for the discrepancy between current and sodium plus lithium flux. Calcium like choline thus is less suitable than Mg as an inert ion.

Table 5 shows results of experiments using a skin bag, which indicate that lithium, like sodium, can be transported by the skin from low concentrations on the outside (epithelial side) to higher concentrations inside (chorion side). This uptake of lithium was independent of movement of water through the skin as is demonstrated in those experiments in which the addition of glucose to the outside Mg-Ringer solution caused, if any, a slow move-

Table 5.

Experiments on skin bags from Rana esculenta. In the first two experiments the skin bags were in their natural position with the epithelial side turned outward, in all the other experiments the epithelial side was turned inward. Column 3 gives the Li concentration in µmol/ml of the chorion side, column 4 on the epithelial side, the first figure at the start, the second at the end of the experiment. Column 5 gives the change in volume of solution in the bag, determined by weighing, the sign refers to the chorion side. The content of the bag at the beginning of the experiment was 2 ml. The 6th column is the mean value of the potential difference. The ΔLi in the 7th column is the increase of Li on the chorion side in the 2 first experiments, the decrease on the epithelial side in the others. Only in the two first experiments the potential difference was occasionally 1—2 mV below zero. The last column gives the solution on the epithelial side, to which the Li-R was added.

Date	Time in hours	Ch.s Li µmol/ml	E.s Li µmol/ml	Δ V gram	P. D. mV	Δ Li µmol	E. sol.
June 25	0 8	4.40 4.96	2.20 »	— 0.008	1	1.08	Mg-R + 60 millimolar dextrose
June 18	0 6	4.40 4.97	2.20 »	0.107	3	1.67	Mg-R
June 25	0 8	4.40 »	2.20 1.15	— 0.020	11	2.08	Mg-R + 60 millimolar dextrose
June 28	0 8	22.0 »	2.20 1.84	— 0.030	10	0.66	Mg-R + 60 millimolar dextrose
June 28	0 8	22.0 »	2.20 1.99	0.020	14	0.46	Mg-R
June 30	0 5	4.40 »	2.20 2.10	0.138	60	0.49	distilled water
June 30	0 5	4.40 »	2.20 2.14	0.174	38	0.48	distilled water

ment of water (Δ V) in the direction opposite to that of lithium. The movement of lithium against an electrochemical potential gradient establishes its active transport by the frog skin.

Discussion.

The experiments shown in Table 1 suggested that the transport of lithium could account for the difference between the net flux of sodium and the short-circuit current.

However it was necessary to exclude the possible active transport of any other ion. This was done by showing that the short-

circuit current (Tables 2 and 3) just equalled the sum of the net flux of sodium plus lithium.

Finally it was demonstrated (Table 5) that lithium could be transported against both an electrical and a chemical gradient; this establishes the active nature of the transport process.

To equate the current with the sum of the sodium and lithium flux, it was necessary to include that portion of the lithium remaining in the skin with the lithium which actually passed through the skin. It is evident that lithium ions which are stored in the skin can only give rise to a current by moving other ions. Possibilities include movement of some cation, *e. g.* potassium from the skin to the inside solution, or the movement of some anion *e. g.* chloride from the inside solution into the skin.

The experiment of March 27 shown in Table 3 illustrates the storage of lithium in the skin. Thus the weight of the skin was 0.142 g, the outside solution contained 3.1 μmol of lithium per ml, hence 0.44 μmol of lithium per 0.142 g of solution. The skin however actually contained 0.65 μmol of lithium so that lithium has been concentrated in the skin. As it is unlikely that the lithium is uniformly distributed through the skin, it is probable that its concentration is much higher in a part of the skin than this average figure would suggest. If lithium were stored only in the epithelial layer its concentration would be at least 5 to 10 times higher than in the outside solution in the experiment cited.

It was of interest to determine whether sodium was similarly concentrated in the skin during its active transport; in order to determine this only the sodium from the outside can be considered. This was conveniently done in the experiments in which sodium influx was measured with Na^{22}. The following data taken from the experiment in Table 2 show the respective contents, derived from the outside solution, of lithium and sodium in the skins.

Experiment	Feb. 10	March 17. I.	March 17. II.	Mean value
Lithium content ...	0.68	0.51	0.54	0.58
Sodium content....	0.09	0.11	0.06	0.09

The ion contents are expressed as μmols and all values have been corrected for the quantity of adhering outside solution by the method described.

It is evident that the quantity of lithium stored is much greater than the quantity of sodium. This difference may be associated

with the greater ionic mobility of the sodium ion. It may also be that lithium which can be readily taken up by the epithelial cells cannot be so readily expelled, as is suggested to be the case with red blood cells (HARRIS and MAIZELS, 1951). It is, in any case, a definite difference in the behavior of the frog skin toward the two ions which otherwise seem to compete on an equal basis in the transport system.

Added in proof. The addition of lithium to the outside of the isolated frog skin may give rise to rhythmic variations of the skin potential and electrical conductance with a frequency of about 0.3 periods per minute (TEORELL, 1954), and they are accompanied by oscillations of the short-circuit current. Control experiments have shown that these oscillations are without any significant influence on the mean value of the short-circuit current, which in the present work is based upon a recording every fifth minute.

Summary.

The transport of lithium ions by the isolated surviving frog skin has been studied. By using the technique of short-circuiting the skin it was first shown that the usual equality of net sodium transport and short-circuit current was disturbed and, secondly, that this disturbance can be accounted for by the quantity of lithium which has passed through the skin plus the quantity which has accumulated in the skin. It was further demonstrated that lithium and sodium compete on an equal basis in the transport process. Finally the ability of the frog skin to move lithium against an electrochemical gradient was shown, thus establishing the active nature of lithium transport.

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From the Histological Department, University of Uppsala, Sweden.

The Fluorescence of the Serum in Rats with Alloxan Diabetes and Cataract.

I. Intensity Determinations.

By

SVEN ELOV BROLIN.

Received 22 December 1954.

The native fluorescence of the eye lens was shown to be strongly increased in severe alloxan diabetes which frequently provoked cataracts (BROLIN 1954). The possibility that the increase could be caused simply by degenerative processes in the lens fibers was not supported by investigations on galactose cataract (BROLIN and KRAKAU 1949) and tetanic cataract (BROLIN 1953). If, however, a fluorescent and toxic substance is allowed to affect the lens its fluorescence will increase as shown by experiments with naphthalin poisoning (BROLIN 1950).

When the increased fluorescence caused by diabetes is considered, the most probable explanation seems to be a deposition in the lens of one or more of fluorescing substances during the course of a developing cataract. Consequently the question will arise whether the supply of fluorescing compounds will be changed by experimental diabetes. The fluorescence of the serum must then be taken into consideration. Different values obtained by estimations of the fluorescence, however, will not necessarily indicate differences in concentration because other factors also may influence the results.

The purpose of the present investigation has been to study:

1) *If the intensity of the serum fluorescence is changed by experimental diabetes.*

2) *If the results of the determinations depend on some other factor than the amount of fluorescing substances.*

In this communication the attention has been confined to the intensity of the fluorescence. Its spectral distribution has also been determined and some small, but perhaps important deviations were observed. This part of the investigation will be published later because in the interpretation special regard must be paid to particular details.

Experimental Procedure and Material.

Diabetes was induced in male rats, of 5—6 weeks age and weighing about 85 g by subcutaneous injection of 0.175 mg alloxan per g body-weight. Previously the rats had been 24 hours without food. The drug was administered as a 1 per cent solution in physiological saline to which for ensuring rapid resorption 0.25 V. R. U. hyaluronidase (Hyalas Leo) had been added per ml. To counteract initial hypoglycaemia about 0.2 g glucose in a 5 per cent solution was given subcutaneously after 2 and 12 hours.

In the course of the experiments which lasted for 5—6 weeks the urine was tested for sugar by clinitest (Ames). When the sugar excretion was high, 0.8—1.6 I. U. zinc-protamine insulin were given subcutaneously once every 24 hours.

The eye lenses were examined in vivo with slit lamp and stereomicroscope after the pupils had been dilated by homatropin. In this way the diabetic rats were separated in groups with and without cataracts. The division of the material is accounted for in Table 1. Double groups were obtained, because the experiments were performed in two different periods.

At the end of the experiments the blood was withdrawn from the rats by the aid of a glass cannula inserted into the abdominal aorta. Chloroform, free from fluorescing impurities was used for narcosis. After centrifugation, the tubes, containing the blood were kept over the night at −2° C and on the following day serum was pipetted off. In this way only slight hemolysis was obtained.

The fluorescence was measured with a spectrophotometer provided with a photocell of electron-multiplier type. The sample was sucked in a quartz tube with an inner diameter of 2 mm, placed in the front of the collimator slit and illuminated by ultraviolet light. The maximum value of each spectrogram was chosen to represent the intensity, the unit of which was a scale division of the galvanometer fed by the photocurrent. In detail the procedure has been described earlier (Brolin 1948, 1953).

Absorption measurements were performed with a Beckman spectrophotometer. To check the influence of pH which of course is not the same as in the living animal, estimations were performed with a glass electrode, permitting an accuracy about 0.02.

Results.

During the experimental period the mean body weight increased much less in the diabetic than in the other groups as proved by statistical calculations. As seen in Table 1 the increase was retarded by diabetes, in the main independent of the aggravation indicated by cataract formation.

The serum fluorescence of the diabetic groups with cataract showed an intensity which was about 50 per cent higher than in the other groups (Table 1). Analysis of variance was used for the statistical treatment of the intensity values, the results being summarized in Table 2. Low variance ratios are obtained by combination of groups with clear lenses, but the ratios increase considerably, when the groups with cataracts are included. No difference between the fluorescence intensity of the simple diabetic groups and the other groups with clear lenses was indicated by the p-value. On the other hand, the probability level corresponding to the possibility that the high fluorescence of the groups with cataracts could be obtained at random from a common homogeneous population was less than 0.1 per cent.

Table 1.

			Number	Intensity of serum fluorescence	Increase of body-weight per cent
Groups with clear lenses.	Normal	N1	20	82	183
		N2	20	93	153
	Alloxan treated but nondiabetic	A1	14	81	161
		A2	11	92	144
	Diabetic	D1	8	94	57
		D2	12	83	95
Groups with cataract	Diabetic	D3	11	142	78
		D4	8	137	38

Student's test was in addition used for calculations, considering only the two kinds of diabetic groups. The common mean value of the two groups with cataract was 140 ± 11.8 which was to be compared with the common value 87 ± 5.3 of the two other

Table 2.

Result of the analysis of variance. The different p-values denote the percentage probability of the variance ratios being exceeded by mere chance. The designation of the groups is explained in Table 1, in which the mean values of the fluorescence intensity is also given.

Combination of groups	Variance Ratio	Probability
N1, N2	0.77	$p > 0.20$
N1, N2, A1, A2	0.49	$p > 0.20$
N1, N2, A1, A2, D1, D2	0.44	$p > 0.20$
N1, N2, A1, A2, D1, D2, D3, D4	4.5	$p < 0.001$
D1, D2, D3, D4	5.7	$0.001 < p < 0.01$

diabetic groups. The difference is 53 ± 12.9 and the t-value 4.1, corresponding to a p-value lower than 0.1 per cent. It seems thus to be well established that the fluorescence of the serum was changed, when diabetes was complicated by cataract formation. The possible influence of some factors on the fluorescence must now be taken into consideration.

Hemolysis.

According to earlier observations (BROLIN 1954) strong hemolysis will depress the intensity values of the fluorescence. Although the method used here successfully limits hemolysis, a continuous control was regarded as justified. The fact that the serum samples should be preserved for further investigations made the control more difficult.

The following procedure and calculations were used to assess approximately the hemoglobin content of the serum samples. With the aid of a pump, air was bubbled through the serum to oxygenate the hemoglobin which was estimated with a Beckman spectrophotometer. As water was used as the blank the E-values corresponding to the peaks of the oxyhemoglobin spectrum were increased by the absorption of the serum. A correction for this serum absorption was calculated as follows.

The measured, apparent absorption (E′-values) may be regarded as the sum of the absorption due to hemoglobin alone (E-values) and the pure absorption of serum. If now this absorption of serum be represented by an arbitrary straight line in the spectral region between 580 and 540 mμ, the E′-values will be obtained as shown in Fig. 1.

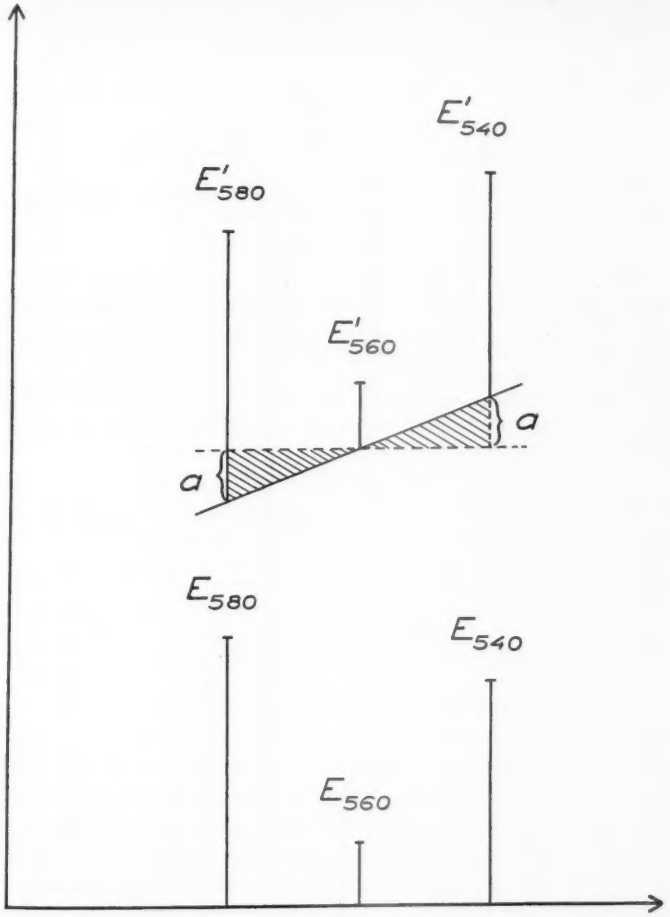

Fig. 1. The E′-values of the sketch are the recorded density figures, which may be regarded as the sum of the actual absorptions of serum and oxyhemoglobin. The inclined line is assumed to represent the serum absorption in the actual spectral region.

Because the shaded triangles of the Fig. 1 are congruent:

$$E'_{580} - E'_{560} = E_{580} - E_{560} - a$$
$$E'_{540} - E'_{560} = E_{540} - E_{560} + a$$

$$E'_{580} - 2E'_{560} + E'_{540} = E_{580} - 2E_{560} + E_{540}$$

Assuming that Beer's law is applicable for the absorption due to hemoglobin alone the following calculations may be performed with the use of c_1, c_2 and k as constants.

$$E_{580} - 2E_{560} + E_{540} = E_{580} - 2c_1 \cdot E_{580} + c_2 \cdot E_{580} = E_{580}\ (1 - 2c_1 + c_2);$$

$$\frac{1}{1 - 2c_1 + c_2}\ (E_{580} - 2E_{560} + E_{450}) = E_{580}$$

If now the E-values in the brackets are substituted by the E′-values as derived above, the formula may be written:

$$E_{580} = k(E'_{580} - 2E'_{560} + E'_{540})$$

The absorption of hemoglobin alone at the wavelength 580 mμ thus may be calculated by using the apparent absorption values, provided that the absorption of serum alone is represented by a straight line. This must be correct in the first two points but at the third one a distance between the real serum absorption curve and the line may exist and give rise to an error. A correction of E'_{540} should thus be performed by adding to it the distance (d), possessing a positive or negative value, whereby the following formula will be obtained.

$$E_{580} = k\ (E'_{580} - 2E'_{560} + E'_{540}) + kd.$$

Provided that d is constant, increasing amounts of hemoglobin will give higher calculated values of E_{580} even if the term k d is neglected. Thus the median of different series may still be compared, the mean on the other hand may not, because the distribution will be altered. In the present investigation the medians were used and k d accordingly neglected, whereby nevertheless an approximation is made, because the variations of d are not taken into consideration.

The positions of the medians are given as percentage figures of the median common to the two normal groups, thus higher figures are indicating increased hemolysis. As seen in Fig. 2 the stronger serum fluorescence of diabetic rats with cataracts should not be explained by weaker hemolysis. Generally the degree of hemolysis seems to be kept low enough to prevent a noticeable influence and as seen in Fig. 3 the absorption peaks of the oxyhemoglobin are not very conspicuous in the long wave part of the spectrogram.

Influence of the blood depôts.

Blood delivered from the depôts may be suspected to constitute different proportions of the sample obtained from smaller and bigger individuals. To check the influence of this possibility, the

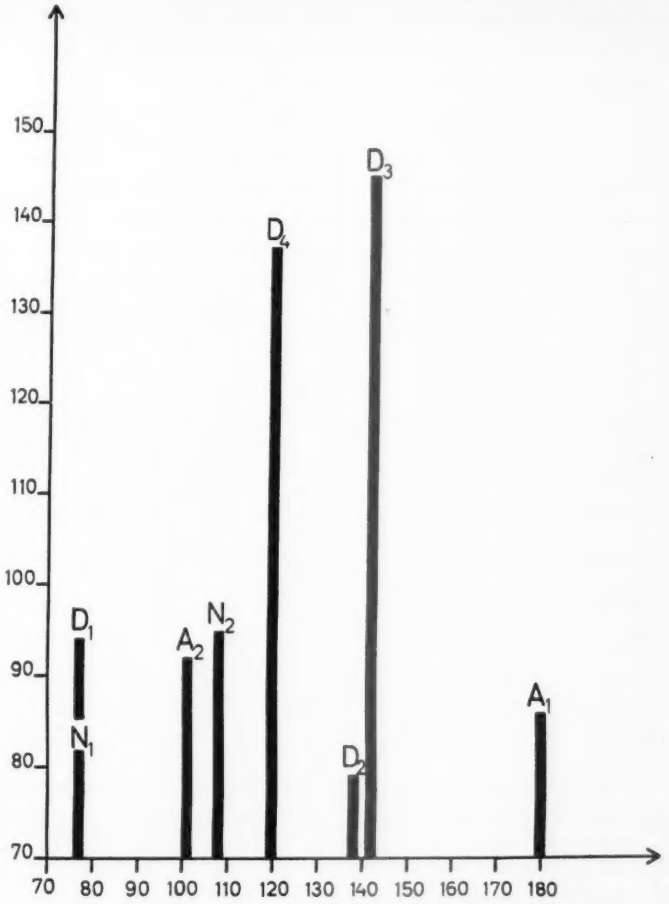

Fig. 2. Result of hemoglobin estimations. The medians of the different groups are given as percentage values of the median, common to the two normal groups. Increasing values of the abscissa thus are indicating higher degrees of hemolysis. The ordinate shows the fluorescence intensity of the different groups, the designation of which being explained in Table 1. As seen high fluorescence intensity is independent of the present degree of hemolysis.

blood was collected in two tubes in 35 cases of the first series. The second tube should reasonably contain a higher proportion of blood from the depôts. The fluorescence intensity of this part

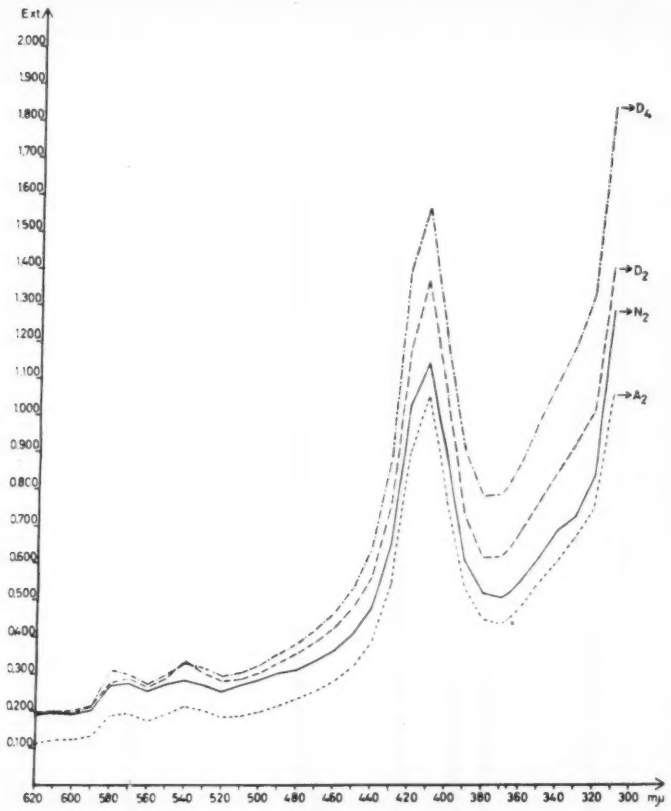

Fig. 3. Mean values of the absorption curves of the groups from the second series. The pathway through the serum sample was 0.5 cm and distilled water was used as blank.

was 9.5 ± 3.3 per cent lower. The possible influence of such a difference on the calculations was considered, but judged as unimportant. Moreover it may be pointed out that differences of body weight scarcely could alter the results, when the diabetic groups with cataracts and clear lenses were compared.

Influence of pH.

Fluorescent determinations are known to be altered by the pH of the solutions. Because of the buffering properties of serum

the pH requirements for accurate determinations should be present. An error may nevertheless be introduced by considerable systematic differences between the groups, thus making pH estimations advisable. Because the experiments were performed at two different periods the mean pH values of the untreated groups were compared with the values of the alloxan-treated groups, see Table 3. The numerical difference was as small as 0.01 in a comparison of the untreated group and the diabetic group with cataract of the first series. The other differences also were small and in no case differed statistically. In consequence of this, displaced pH values of the serum samples have been regarded as a negligible source of error.

Table 3.

A survey of the pH determinations of the serum samples. The normal groups are compared with the different corresponding alloxan treated groups. (The pH meter was calibrated by a standard buffer solution of pH 8.0 when the first series were measured but its value was probably slightly lower at the second period. This source of error is negligible if the comparisons are carried out as below.)

Groups and corresp. pH		Difference
N1—A1	$8.19 \pm 0.03 - 8.26 \pm 0.03$	-0.07 ± 0.04
N1—D1	$8.19 \pm 0.03 - 8.26 \pm 0.03$	-0.07 ± 0.04
N1—D3	$8.19 \pm 0.03 - 8.20 \pm 0.06$	-0.01 ± 0.07
N2—A2	$8.08 \pm 0.01 - 8.10 \pm 0.05$	-0.02 ± 0.05
N2—D2	$8.08 \pm 0.01 - 8.04 \pm 0.08$	$+0.04 \pm 0.08$
N2—D4	$8.08 \pm 0.01 - 8.15 \pm 0.13$	-0.07 ± 0.13

Influence of the absorption in different spectral regions.

As mentioned, absorption measurements, comprising all the material were performed in order to calculate the degree of hemolysis. To facilitate a more complete judgement of the influence of absorption, spectrograms were recorded from all the relevant wavelength, when the experiments were carried out for the second time. Small samples of serum possessing density values of a relative high order should then be investigated. Special cuvettes were designed for this purpose (Brolin 1955). The Beckman spectrophotometer used was provided with a photomultiplier attachment and an adjustable microcuvette holder (Misco, Berkeley). Spectrograms, representing the different groups

are given in Fig. 3. The exiting ultraviolet light is mainly found at the wavelength 366 mμ. The measured intensity of fluorescence should be diminished by absorption in this spectral region. The lower fluorescence intensity of the groups with clear lenses cannot however be explained by such an absorption, see Fig. 3. Nor can the strong fluorescence of the diabetic group with cataract depend on a lower absorption in the visible spectral region.

Discussion.

High intensity values seem to indicate an increased concentration of fluorescing substances in the serum, because the experiments did not support other alternatives of interpretation.

The investigation has sustained the assumption that fluorescing compounds are deposited in the lens when a cataract is brought about by experimental diabetes. It may especially be pointed out that an increased fluorescence of the serum could not be detected in series with diabetes where the lenses remained clear. A special question, not falling in the scope of this investigation is, whether the fluorescing compounds are toxic and in this way taking part in the cataract formation. An experimental consideration of this question has been planned.

Summary.

The theory has been presented that fluorescing compounds are deposited in the lens at the formation of an experimental diabetic cataract. The increase of the lens fluorescence was previously stated. A marked and significant increase of the serum fluorescence has been recorded in rats with alloxan diabetes, complicated by cataracts. No change of the fluorescence intensity could be observed in other experimental groups comprising diabetes with clear lenses and alloxan treatment, not giving rise to diabetes. Special regard was paid to hemolysis, different emptied blood depôts, pH and absorption in different spectral regions of the serum samples, but the influence of these factors did not constitute any appreciable source of error.

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From the Histological Department, University of Uppsala, Sweden.

A Microcuvette Designed for Absorption Measurements of Biological Fluids with High Density Values.

By

SVEN ELOV BROLIN.

Received 22 December 1954.

Three general possibilities present themselves, if spectrophotometric determinations of high density values are to be carried out. These alternatives consist in a dilution of the sample, the choice of an appropriate blank with high density and the use of special cuvettes. If, however, biological fluids are to be estimated, it may be desirable to avoid dilution or difficult to find a convenient blank. The remaining possibility of using cuvettes with a short liquid pathway of light also will be limited by the fact that accurate such cuvettes are not commercially easily obtainable. In the course of an investigation on the properties of diabetic serum (BROLIN 1955) microcuvettes according to the sketch of Fig. 1 were constructed.

The cuvettes are turned out of a hard and transparent plastic (methyl methacrylate resin). Thus all cross-sections have circular surfaces. The end surfaces as well as the air pathways are blackened with cellulose laquer to prevent undesired light from reaching the photocell. As seen in Fig. 1, the dimensions of the air pathways and the liquid chamber are chosen to prevent internal reflexions. The quartz windows are fixed with a special cement for joining glass to plastic.

When the liquid chamber has been filled and the cover piece is screwed on, the air is easily displaced through the outlet. The screw of the cover may be tightened more or less, thus permitting

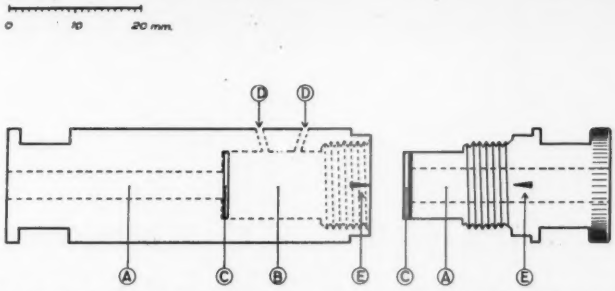

Fig. 1. To the left main part of a cuvette with the sample chamber. To the right the cover with its inserting piece, the length of which may be matched to one of the two air outlets.
A) Air pathways.
B) Liquid pathway.
C) Quartz windows.
D) Outlets for air bubbles.
E) Engraved marks which shall coincide, when the cover is screwed into position.

a fine adjustment of the chamber depth which is indicated by the marks engraved on both the screw inserting piece and the main piece. Depending on the length of the inserting piece 1.0 or 0.5 ml of liquid was used. It might be possible to diminish the volume of the chamber by an auxiliary piece of a cylinder provided with a central channel.

The cuvettes were placed in an adjustable microcuvette holder (MISCO, Berkeley), manufactured for the Beckman spectrophotometer. When calibration was performed by using a pair of cuvettes filled with distilled water, the error of the density values was less than 0.5 per cent in most of the spectral regions. Serum spectrograms recorded with the described cuvettes are presented in a following publication (BROLIN 1955).

Summary.

The design of cuvettes for measuring small samples of biological fluids with high density values is given.

The technical assistance of Mr. R. Kikas in the construction of the microcuvettes is gratefully acknowledged.

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From The Department of Obstetrics and Gynecology B, Rigshospitalet and The Department of Pharmacology, University of Copenhagen.

Uric Acid in Blood and Urine of Infants.

By

HEMMING POULSEN.

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It is a long-established fact that the urine of the newborn contains large amounts of uric acid. SCHLOSS and CRAWFORD as early as in 1911 demonstrated a high incidence of uric acid deposits in the renal pyramids of infants. They found such concretions in about 40 % of all infants within the first few days of life. The authors examined 20 infants and found a 24-hour uric acid excretion of from 18 to 27 mg per kg during the first 4 days of life. Then followed a 24-hour excretion of about 10 mg per kg the next few days.

No such investigations have been made on infants since the introduction of a new specific and accurate method for uric acid determination. As, moreover, the above writers did not collect urine by catheterisation, we have thought that renewed investigations might be of interest.

At the same time we have determined the uric acid concentrations in blood from the umbilical cord and in the amniotic fluid, as well as in plasma from the infants 24, 48, and 72 hours after birth. For comparison we have measured the uric acid excretion in a smaller series of older experimental subjects: two infants (aged 3 and 5 years) as well as three adult men and five adult women.

Technique.

Methods of Analysis.

The uric acid determinations were carried out by the enzymatic-spectrophotometric method indicated by KALCKAR (1947), which is

based on the view that the uric acid concentration is proportional to the extinction fall at 292.5 mμ after addition of uricase. PRÆTORIUS has modified the method for clinical serial analyses (1949) and it was subsequently improved by PRÆTORIUS and POULSEN (1953).

Owing to the high uric acid concentrations the determinations in the *urine* were made with 25 μl of urine diluted with a buffer of pH 9.3 to 10 ml.

The uric acid concentrations in *plasma* were determined in two ways, dependent on the amount of plasma available. For small quantities of plasma a special modification elaborated by the authors of the method for small volumes (1953) was employed, *e. g.* 25 μl of plasma diluted with 1 ml of buffer. Where larger amounts of plasma were available we generally used 268.5 μl of plasma diluted with buffer to 10 ml.

Experimental Methods.

The investigation comprised 15 infants, from whom urine was collected for 3 to 5 days and blood samples were taken the first 3 days. Liquor amnii was collected during the process of delivery, and blood from the umbilical cord immediately after birth. Next catheter à demeure was inserted, Tiemann's catheter No. 7—8 for boys and Nelaton's catheter No. 11—13 for girls. The catheter was fixed with adhesive plaster.

The infants were laid on one side with the legs and body fixed just enough to avoid tearing out of the catheter. The urine was collected for 24-hour periods the first 3 to 5 days, with the first period reckoned from the hour of birth. The urine specimens were all analyzed the same day as the period of collection was concluded. The blood samples were taken by heel pricking on the first, second, and third days, independently of the hour of birth, generally between 11 and 12 in the morning.

Thirteen of the infants had been born quite naturally, while in two cases (Nos. 3 and 12) delivery with forceps had been necessary owing to secondary inertia. The series of infants examined comprised eight boys and seven girls. Two had pronounced jaundice (Nos. 6 and 13) and one mild jaundice (No. 14).

All the infants were given 100 mg sulfamethizol (lucosil R) prophylactically in the morning and at night. One infant developed coliuria with fever (No. 9), which subsided under aureomycin treatment. The others had sterile urine at the conclusion of the experiment. All the infants received artificial nourishment. For the sake of comparison we examined two infants aged 3 and 5 years and eight adults between the ages of 26 and 57.

The 24-hour urine specimens were collected in the way that the 24-hour period was reckoned from the time after the first emptying of the bladder till the corresponding point of time the next day. Care was taken that the first withdrawal of urine was carried out at the same hour on each experimental day. The urine specimens were analyzed on the day of their collection.

Table 1.

The diuresis, the uric acid concentration and the amount of excreted uric acid per kg body weight in the first three to five 24-hour periods after birth.

Infant	1. day			2. day			3. day			4. day			5. day		
	Diuresis in ml	Uric acid		Diuresis in ml	Uric acid		Diuresis in ml	Uric acid		Diuresis in ml	Uric acid		Diuresis in ml	Uric acid	
		µg/ml	µg/kg		µg/ml	µg/kg		µg/ml	µg/kg		µg/ml	µg/kg		µg/ml	µg/kg
1.....	37.5	1,920	22.6	38	1,766	21.3	105	684	22.8	—	—	—	—	—	—
2.....	67	1,098	19.9	68	1,186	21.8	116	679	21.3	—	—	—	—	—	—
3.....	55	1,247	18.8	111	700	21.3	91	662	16.3	114	750	23.5	—	—	—
4.....	29.5	1,457	10.0	63	1,418	20.8	136	662	20.9	—	—	—	—	—	—
5.....	19	2,091	12.8	41	1,545	20.4	26	772	—	—	—	—	—	—	—
6.....	11.5	1,923	6.7	17	2,212	11.4	36	1,790	19.5	136	761	31.4	105	734	23.3
7.....	26	2,036	14.9	29	1,909	15.6	95	646	17.3	108	486	14.8	—	—	—
8.....	26	1,971	13.7	25	1,131	7.5	68	618	11.2	131	348	12.2	118	193	6.1
9.....	47	1,225	19.2	20	1,032	6.9	29	1,258	12.2	—	—	—	—	—	—
10.....	19	2,207	11.5	19	2,179	11.4	49	1,473	19.8	101	546	15.4	—	—	—
11.....	20	1,659	7.9	38	1,299	11.8	34	1,101	8.9	—	—	—	—	—	—
12.....	14	2,149	8.5	47	1,686	22.3	77	1,026	22.2	79	1,026	22.8	152	338	14.5
13.....	21	2,320	13.2	37	1,192	11.9	33	993	8.9	31	1,584	13.0	54	1,101	16.0
14.....	22	1,552	10.3	25	1,347	10.2	66	703	14.1	149	381	17.2	148	381	17.1
15.....	106	1,090	41.3	88	660	20.8	124	532	23.5	171	344	21.0	223	226	18.0
Average	35	1,730	15.4	44	1,417	15.7	72	893	15.9	113	692	19.0	133	496	15.8

This series comprised two girls, three men, and five women. None of these subjects had any signs or symptoms of disease and have been in good health for a year after the experiment. The diet of the adult experimental subjects was an ordinary one containing about 100 g protein daily.

Results.

The newborn infants excrete only endogenous uric acid (purine-free diet). The 24-hour excretion amounts to about 16 mg/kg (Table 1). Adults on an ordinary diet containing about 100 g protein daily excrete about 8 mg/kg during 24 hours (Table 3). Roughly about half of this is exogenous. In other words, the endogenous 24-hour uric acid excretion of adults can be set at about 4 mg/kg. Newborn infants thus excrete about four times more endogenous uric acid per kg than adults.

The amount of urine produced increases from day to day. It is remarkable that such high uric acid concentrations are found in the urine the first 48 hours (Table 1). The plasma concentration falls somewhat the first few days, and is generally a little higher than that normally seen in adults (Table 2).

Table 2.

The plasma uric acid concentration during the first three days after birth and the uric acid concentration in the amniotic fluid and in blood from the umbilical cord.

Infant	Plasma			Liquor amnii	Blood from the umbilical cord
	1. day μg/ml	2. day μg/ml	3. day μg/ml	μg/ml	μg/ml
1.....	50.0	68.8	57.5	53.5	37.5
2.....	50.0	36.5	39.0	53.5	44.0
3.....	41.4	54.5	51.5	94.5	75.0
4.....	45.0	53.5	50.5	99.0	79.0
5.....	50.5	55.0	56.0	57.0	61.5
6.....	45.2	47.4	48.0	68.6	50.0
7.....	65.5	65.0	59.0	65.0	57.5
8.....	68.0	70.0	67.0	65.0	65.0
9.....	61.0	58.0	54.5	71.5	64.0
10.....	56.0	51.5	45.2	65.0	63.5
11.....	50.1	—	51.2	69.1	55.5
12.....	69.4	44.6	41.8	112.1	81.0
13.....	59.5	53.9	49.5	77.9	68.0
14.....	53.4	49.5	45.2	—	67.5
15.....	47.8	33.6	24.2	69.1	55.5
Average..	54.2	53.0	49.3	72.3	61.1

Table 3.

The urinary uric acid excretion/kg body weight in consecutive 24-hour periods by the adult man, woman and 3—5 year child.

	1. day mg/kg	2. day mg/kg	3. day mg/kg	4. day mg/kg	5. day mg/kg	6. day mg/kg	7. day mg/kg	Average mg/kg
Man	7.6	8.0	6.8	8.6	8.3	7.4	6.9	
—	7.4	6.9	6.0	7.1	8.9	7.5	6.3	8.1
—	8.3	9.4	11.9	9.9	7.4	7.7	6.3	
Woman	6.4	7.8	9.7	8.1	8.8	7.4	9.1	
—	8.0	7.7	7.4	8.5	8.4	7.7	7.9	
—	8.6	9.4	8.2	8.9	9.1	7.7	—	8.0
—	7.7	6.3	6.6	7.0	7.1	6.9	—	
—	7.5	7.3	7.3	9.6	16.0	6.8	—	
Child	10.3	9.5	11.5	11.8	10.9	9.6	—	11.0
—	9.6	12.8	9.7	11.2	10.8	10.0	—	

On the basis of the average urine flow, as well as the uric acid concentrations in plasma and urine the clearance of uric acid has been found to be of the order of 1 ml per minute, *i. e.* about 0.3 ml per minute per kg. The uric acid clearance of adults is about 0.1 ml per minute per kg.

The uric acid concentration in liquor amnii is higher than that in plasma from the umbilical cord blood (Table 2). There is, however, a certain parallelism, so that high values in one are attended by high values in the other.

Discussion.

The uric acid excretion in newborn infants was here found to be somewhat lower than indicated by SCHLOSS and CRAWFORD. The difference is probably due to the fact that the figures obtained with the method employed by SCHLOSS and CRAWFORD (FOLIN 1914) were too high, presumably because the reagents used were reduced by other substances than uric acid.

That the 24-hour excretion of uric acid per kg is greater in infants than in adults is a fact to be expected, if — as is generally accepted — we take the uric acid excretion in man to indicate the intensity of the nuclein metabolism.

Summary.

The 24-hour excretion of endogeneous uric acid in newborn infants was found to be about 16 mg/kg and in adults about 4 mg/kg. The average concentration of uric acid in blood from the umbilical cord was 61 μg/ml and in the amniotic fluid 72 μg/ml.

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From the University Institute of Physical Chemistry and the Biological Institute, Carlsberg Foundation, Copenhagen, Denmark.

On a Fibrinolytic Enzyme in the Perilymph of Sharks.

By

C. E. JENSEN.

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The existence of a fibrinolytic system in certain fluids occurring in various small glandular ducts in the animal organism was proved by MACFARLANE and PILLING (1947), BJERREHUUS (1952) and ASTRUP and STERNDORFF (1952, 1953). The function of these fibrin splitting enzymes might be to counteract the obstruction of these minute ducts by clotted fibrin. The present investigation was undertaken to ascertain whether the fluids of the inner ear might contain a fibrinolytic mechanism of this sort.

Material.

The piked dogfishes (Acanthias vulg.) were delivered on ice and had been dead for about 50 hours when the peri- and endolymph were withdrawn, and the fluids were kept at 5° C for five days before the investigations began. A small drop of toluene was added in order to prevent bacterial growth. The perilymph appeared as a rather thin watery and homogeneous fluid just after its withdrawal from the perilymphatic space. Upon standing, a "cloud formation" could sometimes be seen and — more rarely — a true coagulation of the whole mass of fluid was observed. These coagula dissolve spontaneously in the course of 12 to 24 hours. The endolymph is highly viscous, jellylike and transparent.

Fig. 1. Fibrin plate incubated 18 h. with perilymph at 20° C.

Method.

In order to test the possible occurrence of fibrinolytic activity in the labyrinthine fluids the fibrin plate method of ASTRUP and MÜLLERTZ (1952) was used.

The extent of digestion of fibrin, defined as the product of two mutually perpendicular diameters (the thickness of the plates being practically constant), was used as the criterion for fibrinolytic activity. The added amounts of endolymph were only approximately equal because of its jellylike behaviour. In some experiments three drops were placed separately on each dish, whereas in others only one was used in order to economize on the scanty materials.

Experiments and Results.

In the first experiment 3 drops of the perilymph and 4 drops of the endolymph were placed at separate points on the standard fibrin plates. After incubation for 18 hours at 37° C three lysed zones of 169, 156 and 156 mm², respectively, were seen on the plate incubated with perilymph. The plate with endolymph did

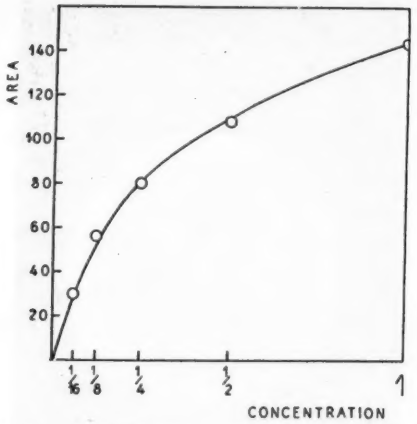

Fig. 2. Relation between activity and concentration.
Ordinate: Area in square mm.
Abscissa: Concentration in units of the original perilymph (= 1).
Incubation time: 18 h.
Temperature: 20° C.

not reveal tryptic activity, although care was taken to ensure contact between the highly viscous, jellylike endolymph and the fibrin plate.

In order to test whether the fibrinolytic activity of the perilymph might be ascribed to an enzyme or to a plasminogen activator, the experiments were performed after incubation on fibrin plates previously heated at 85° C for 45 minutes, whereby their plasminogen is destroyed. However, a lysis was still observed; the areas of the lysed zones were smaller (72, 64, 56 mm²) than in a parallel experiment on a normal plate (81, 60, 73 mm²), but this might be due to a partial denaturation of the fibrin.

The addition of a surplus amount of plasminogen to standard plates did not cause any significant increase of the lysed zones. Neither did addition of streptokinase and fibrinokinase cause any enhancement of the fibrinolytic activity.

In order to investigate the relation between activity and concentration, drops of perilymph diluted to 4 different concentrations with a diethyl barbiturate buffer (ionic strength: 0.05, pH 7.8) in 0.9 per cent saline were placed on standard fibrin plates and incubated at 20° C for 18 hours. A pronounced activity was found when the perilymph was diluted eight times, and even at

a dilution to sixteen times a lysis could still be observed. With increasing concentration of enzyme the areas of the lysed zones increased as shown in Fig. 2.

After incubation for 18 hours at 5°, 9° and 20° C, respectively, followed by additional incubation at 20° C for periods up to 72 hours, the areas of the lysed zones progressively increased. However, after preliminary incubation at 37° C the area remained unchanged in size when subsequently incubated at 20° C (Table 1). With increasing incubation time the differences in areas of the lysed zones digested at 5°, 9° and 20° C are reduced. It was impossible to follow the reaction for more than 72 hours, as the zones began to merge and a bacterial growth could not be excluded.

Table 1.

Relation between temperature, incubation time and activity.

Temp. °C	Incubation time 18 h. Average Area of 3 lysed zones mm²	Temp. °C	Incubation time 48 h. Average area mm²	Incubation time 72 h. Average area mm²
5	44	20	422	589
9	51	20	439	600
20	225	20	484	629
37	82	20	87	90

To study the inactivation of the enzyme by temperature, the perilymph was kept at 37° C for various intervals of time and the activities measured after incubation at 20° C for 24 hours and 48 hours, respectively. The "half-life"-period was about 2 hours and the activity was completely lost after incubation for 12 hours at 37° C (Table 2).

Table 2.

Inactivation of the enzyme after incubation at 37° C.

Incubation time in h. at 37° C	0	1/4	1/2	2	4	12
"Area" after 24 h. mm²	306	210	196	154	49	0
"Area" after 48 h. mm²	460	342	306	238	81	0

Summary.

The perilymph of the piked dogfish contains a fibrinolytic enzyme which is destroyed by incubation for 12 hours at 37° C. The enzyme has its maximum activity at 20° C.

I am indebted to Dr. TAGE ASTRUP, Biological Institute, Carlsberg Foundation, to Professor J. A. CHRISTIANSEN and to Dr. THURE VILSTRUP for valuable advice and encouragement. The photograph is due to A. E. LANSNER, Chemical Engineer.

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